From DIVISION OF MOLECULAR NEUROBIOLOGY
DEPARTMENT OF MEDICAL BIOCHEMISTRY AND BIOPHYSICS
Karolinska Institutet, Stockholm, Sweden

CALCIUM SIGNALING IN NEUROGENESIS:

REGULATION OF PROLIFERATION, DIFFERENTIATION AND MIGRATION OF NEURAL STEM CELLS

Paola Rebellato

Karolinska Institutet

Stockholm 2013

Published by Karolinska Institutet. Printed by Åtta.45 Tryckeri AB, Sundbyberg

ISBN 978-91-7549-359-6

Cover: picture of human neuroephitelial cells loaded with the calcium dye Fluo-3/AM and captured using an upright fluorescence microscope equipped with an 20× 1NA lens (Carl Zeiss). Picture modified by Carlos Villaescusa.

To my family

Division of Molecular Neurobiology,

Department of Medical Biochemistry and Biophysics

CALCIUM SIGNALING IN NEUROGENESIS: REGULATION OF PROLIFERATION, DIFFERENTIATION AND MIGRATION OF NEURAL STEM CELLS

AKADEMISK AVHANDLING

som för avläggande av medicine doktorsexamen vid Karolinska Institutet offentligen försvaras i Hillarpsalen, Institutionen för Neurovetenskap, Retzius väg 8, Karolinska Institutet

Fredagen den 13 December, 2013, kl 10.00

av

Paola Rebellato

Huvudhandledare:
Assoc. Prof. Per Uhlén
Karolinska Institutet
Department of Medical Biochemistry and Biophysics

Bihandledare:
Professor Ernest Arenas
Karolinska Institutet
Department of Medical Biochemistry and Biophysics

Dr. Seth Malmersjö
Stanford university
Department of Chemical and Systems Biology

Ass. Prof. J. Carlos Villaescusa
Karolinska Institutet
Department of Medical Biochemistry and Biophysics

Fakultetsopponent:
Professor Michael J. Berridge
The Babraham Institute
Laboratory of Molecular Signalling

Betygsnämnd:
Professor Gilberto Fisone
Karolinska Institutet
Department of Neuroscience

Professor Erik Gylfe
Uppsala University
Biomedical Center

Professor Jonas Muhr
Karolinska Institutet
Department of Cell and Molecular Biology

Stockholm 2013

ABSTRACT

The calcium ion (Ca^{2+}) is a highly versatile and ubiquitous signaling messenger in all cell types. Signal transduction occurs through changes in the cytosolic Ca^{2+} concentration after the opening of Ca^{2+} channels in the plasma membrane (PM) and endoplasmic reticulum (ER). The difference in Ca^{2+} concentration between the extracellular space and the cytosol is large, around 10,000 fold, creating a steep gradient that causes Ca^{2+} to rapidly flow into the cell. Signaling via Ca^{2+} is fundamental for triggering numerous vital processes in the cell, ranging from fertilization to cell death. Calcium signaling is also critical for regulating neurogenesis in various ways, some of which have been explored in this work.

Proliferation of neural progenitors is dependent on spontaneous Ca^{2+} activity that occurs in small-scale networks. Ca^{2+} activity is correlated with electrical activity both *in vitro* and *in vivo* and depends on connexin 43 gap junction and PM channels. Differentiation of neural progenitors is also regulated by Ca^{2+} signaling. We have found that T α1h voltage-dependent Ca^{2+} channels promote spontaneous Ca^{2+} activity and direct the differentiation of human neuroepithelial stem cells towards neurons, depending on caspase-3 enzymatic activity. These results were confirmed with T α1h knockout mice that showed a decreased number of neurons in the dorsal cortex. Neuronal migration also depends on Ca^{2+} signaling. We demonstrated that glial derived neurotrophic factor (GDNF) stimulates a Ca^{2+} response through the activation of the receptor tyrosine kinase (RET). The subsequent downstream signaling cascade includes phospholipase Cγ, which binds to RET Tyr1015. Mutating RET at Tyr1015 inhibits neuronal progenitor migration towards the cortical plate. We also showed that neurogenesis was altered by the addition of non-cytotoxic concentrations of polychlorinated biphenyls that disrupt spontaneous Ca^{2+} activity. Polychlorinated biphenyls are common food contaminants. In addition, methyl mercury, another food contaminant, disrupts neuronal differentiation in the opposite direction. Altogether, these data demonstrate the huge impact of Ca^{2+} signaling on the development of the embryonic brain.

To conclude, we have analyzed Ca^{2+} signaling during three critical steps of neurogenesis: proliferation, differentiation, and migration. All of these processes are known to be dependent on Ca^{2+}. A deeper understanding of how Ca^{2+} regulates such different physiological processes is crucial for the field of regenerative medicine, in which control of the expansion and differentiation of neural stem cells can increase the production of neuronal cells *in vitro* for use in cell replacement therapies.

LIST OF PUBLICATIONS

I. Malmersjö S*, REBELLATO P*, Smedler E*, Planert H, Kanatani S, Liste I, Nanou E, Sunner H, Abdelhady S, Zhang S, Andäng M, El Manira A, Silberberg G, Arenas E, Uhlén P (2013) **Neural progenitors organize in small-world networks to promote cell proliferation.** Proc Natl Acad Sci U S A. 2013 Apr 16;110(16):E1524-32

II. REBELLATO P, Kanatani S, Villaescusa C, Falk A, Arenas E, Uhlén P **T α1h-Channel Dependent Spontaneous Ca^{2+} Activity Regulates Neuronal Differentiation Through Caspase-3.** Manuscript

III. Tofighi R*, Wan Ibrahim WN*, REBELLATO P, Andersson PL, Uhlén P, Ceccatelli S (2011) **Non-dioxin-like polychlorinated biphenyls interfere with neuronal differentiation of embryonic neural stem cells.** Toxicol Sci. Nov;124(1):192-201

IV. Lundgren TK*, Nakahata K*, Fritz N*, REBELLATO P, Zhang S, Uhlén P (2012)
RET PLCγ phosphotyrosine binding domain regulates Ca^{2+} signaling and neocortical neuronal migration. PLoS One. 2012;7(2):e31258

*these authors contributed equally to the work

PUBLICATIONS NOT INCLUDED IN THE THESIS

Malmersjö S, REBELLATO P, Smedler E, Uhlén P (2013) **Small-world networks of spontaneous Ca^{2+} activity.** Commun Integr Biol. Jul 1;6(4):e24788.

Wan Ibrahim WN, Tofighi R, Onishchenko N, REBELLATO P, Bose R, Uhlén P, Ceccatelli S (2013) **Perfluorooctane sulfonate induces neuronal and oligodendrocytic differentiation in neural stem cells and alters the expression of PPARγ in vitro and in vivo.** Toxicol Appl Pharmacol. May 15;269(1):51-60

Ibarra C, Vicencio JM, Estrada M, Lin Y, Rocco P, REBELLATO P, Munoz JP, Garcia-Prieto J, Quest AF, Chiong M, Davidson SM, Bulatovic I, Grinnemo KH, Larsson O, Szabadkai G, Uhlén P, Jaimovich E, Lavandero S (2013) **Local control of nuclear Ca^{2+} signaling in cardiac myocytes by perinuclear microdomains of sarcolemmal insulin-like growth factor 1 receptors.** Circ Res. Jan 18;112(2):236-45

REBELLATO P, Islam S (2013) **[6]-shogaol induces Ca^{2+} signals by activating the TRPV1 channels in the rat insulinoma INS-1E cells.** Manuscript accepted for publication in Journal of the Pancreas

Contents

LIST OF ABBREVIATIONS

AIF	Apoptosis-Inducing Factor
AMPA	α-Amino-3-hydroxy-5-Methyl-4-isoxazolePropionic Acid)
ATP	Adenosine TriPhosphate
Ca^{2+}	Calcium
$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
CaM	CalModulin (CALcium-MODULated proteIN)
CaMK	Ca^{2+} /CalModulin dependent protein Kinase
cAMP	Cyclic Adenosine MonoPhosphate
CARE	Ca^{2+} Response Element
CBP	CREB Binding Protein
CCE	Capacitative Ca^{2+} Entry
CDK	Cyclin-Dependent Kinase
CICR	Ca^{2+} Induced Ca^{2+} Release
CNG	Cyclic Nucleotide-Gated
CRE	cAMP Response Element
CREB	cAMP Response Element Binding
CREST	Ca^{2+} RESponsive Transactivator
DAG	Diacylglycerol
DISC	Death-inducing signaling complex
ER	Endoplasmic Reticulum
ES cells	Embryonic Stem cells
FAD	Flavin Adenine Dinucleotide
FGF	Fibroblast Growth Factor
GDNF	Glial-Derived Neurotrophic Factor
GFP	Green Fluorescent Protein
GPCR	G protein-coupled receptor
HCN	Hyperpolarization-activated cyclic nucleotide-gated
$InsP_3$	Inositol 1,4,5-trisphosphate
$InsP_3R$	Inositol 1,4,5-trisphosphate Receptor

MAPK	Mitogen-Activated Protein Kinase
NCCE	Non Capacitative Ca^{2+} Entry
NCX	Na^{2+}/Ca^{2+} Exchanger
NF-AT	Nuclear Factor of Activated Cells
NMDA	N-methyl-D-aspartate
NSC	Neural Stem Cells
NS cells	Neuroephitelial Stem cells
Orai	Calcium release-activated calcium channel protein 1
PM	Plasma Membrane
PCB	Polychlorinated Biphenyls
PKC	Protein Kinase C
PLC	Phospholipase C
PMCA	Plasma Membrane Ca^{2+} ATPase
RET	REarranged during Transfection
ROCs	Receptor Operated Channels
RTK	Receptor Tyrosine Kinase
RyR	Ryanodine receptor
SERCA	Sarco/Endoplasmatic Reticulum Ca^{2+} -ATPase
SMOCs	Second Messenger Operated Ca^{2+} Channels
SOCs	Store Operated Channels
SOCE	Store Operated Ca^{2+} entry
SPCA	Ca^{2+} ion-transporting P-type ATPase
STIM	Stromal Interaction Molecule
TH	Tyrosine Hydroxylase
VOCs	Voltage Operated Channels
VZ	Ventricular Zone
TRP	Transient Receptor Protein
TTX	Tetrodotoxin
VDCC	Voltage Dependent Ca^{2+} Channels (same as VOC)

1 INTRODUCTION

1.1 Ca^{2+} SIGNALING

An experiment performed 130 years ago by Sydney Ringer marked the beginning of the calcium (Ca^{2+}) signaling field. Ringer was studying contraction using isolated rat hearts suspended, under his admission, in tap water. The hearts contracted beautifully in London's hard water. When Ringer decided to increase the quality of his experiment and use distilled water, hearts gradually stopped to contract. Ringer had to add Ca^{2+} salts to maintain cardiac contraction. Thus, this experiment, which by today's standards was deeply flawed, instigated the study of Ca^{2+} signaling.

1.1.1 Ca^{2+} signaling toolkit

The cytoplasmic Ca^{2+} concentration in a healthy cell is approximately 100 nM, while the extracellular concentration is 10,000–20,000 fold higher (between 1 and 2 mM), thus creating a strong gradient across the plasma membrane (PM). When channels on the membrane are open, Ca^{2+} can passively diffuse into the cells and increase the cytoplasmic concentration to approximately 1 µM. Intracellular compartments such as the endoplasmic reticulum (ER) or the mitochondrion maintain specific Ca^{2+} concentrations of 0.2–1 mM and 0.1–10 µM, respectively.

Ca^{2+} homeostasis in a cell is regulated by a multitude of Ca^{2+} regulators that are highly coordinated to control spatial and temporal changes in Ca^{2+} concentration. The set of all Ca^{2+} regulators is called the Ca^{2+} signaling toolkit. Ca^{2+} signaling can then be divided in to four processes:

Encoding: This process involves the activation of the Ca^{2+} signaling toolkit in response to intra- or extracellular stimuli. For example, membrane depolarization of excitable cells leads to the opening of the voltage-operated Ca^{2+} channel (VOCs) in the PM. On the endoplasmic reticulum (ER), 1,4,5-trisphosphate (InsP3) activates the $InsP_3$ receptor to release Ca^{2+} stored in the ER.

ON mechanism: Elevation of intracellular Ca^{2+} can be generated from the extracellular space or from the intracellular Ca^{2+} stores (i.e., the ER) after the opening of the channels. Channels in the PM and in

ER open in response to different stimuli, such as changes in voltage, binding of an agonist, or release of calcium, etc. (Table 1).

ON mechanisms that increase intracellular calcium levels		
Channels	**Location**	**Example**
Voltage-operated channels (VOCs)	Plasma membrane	L, P/Q, N, R, T type
Receptor-operated channels (ROCs)	Plasma membrane	NMDA, AMPA, ATP receptors
Second messenger operated channels (SMOCs)	Plasma membrane	CNG, HCN
Store-operated Ca^{2+} channels (SOCs)	Plasma membrane	Orai1, Orai2, Orai3
Transient Receptor Potential (TRP) ion channels	Plasma membrane	TRPC1-7, TRPV1-6, TRPM1-8
Inositol 1,4,5 triphosphate receptor	Endoplasmic Reticulum	InsP3R1-3
Ryanodine receptors	Endoplasmic Reticulum	RyR1-3
Store-operated Ca^{2+} channels (SOCs)	Endoplasmic Reticulum	STIM1, STIM2

Table 1: On mechanism channels.

Decoding: translation of increased levels of Ca^{2+} into a physiological process. Ca^{2+} is an ion and it is the only second messenger that does not undergo any structural or molecular changes to initiate signaling. The binding of Ca^{2+} to a calcium-binding protein can modulate the conformation and charge state of such proteins with consequences on their function. These Ca^{2+} binding proteins can be a Ca^{2+} sensor or Ca^{2+} buffer, but only proteins in the first category are directly involved in signaling, activating different cellular processes after Ca^{2+} binding. Ca^{2+} buffers undergo only minor conformational changes and consequently function only as buffer or transporters. Through its four EF-

hands that can bind Ca^{2+}, calmodulin (CaM) is one of the most global sensor proteins, and interacts with more than 100 target proteins that regulate a variety of different processes, such as gene transcription or muscle contraction. The most common Ca^{2+} binding proteins are listed in Table 2

Ca^{2+} sensors	Calmodulin, TroponinC, Synaptotagmin, S100, Annexin, Neuronal Ca^{2+} sensor, Hippocalcin, DREAM
Ca^{2+} buffers	**Cytosolic**: CalbindinD-28K, calbindin-D9k, Calretinin, Parvalbumin **ER/SR**: Calnexin, calreticulin, GRP 78

Table 2: Intracellular Ca^{2+} binding proteins.

Each type of cell possesses different Ca^{2+} regulators whose expression can be remodeled depending on the need. Many enzymes or transcription factors are indirectly regulated by calcium, such as those reported in Table 3.

Ca^{2+} sensitive enzymes	CAMK, myosin light chain kinase, phosphorylase, Adenylyl cyclase, PYK2, PKC, nitric oxide synthase, calcineurin, phosphodiesterase
Ca^{2+} sensitive transcription factors	NFAT, CREB,CBP

Table 3: Enzymes and transcription factors indirectly sensitive to Ca^{2+}.

OFF mechanism: The removal of Ca^{2+} is necessary for the restoration of the basal Ca^{2+} level in the cytoplasm. Long-term increases in Ca^{2+} are toxic for the cell, so it is fundamental to have an efficient and rapid Ca^{2+} removal system. Ca^{2+} pumps and exchangers are located on the PM and the ER and are summarized in Table 4.

OFF mechanisms that decrease intracellular calcium levels		
Pumps and exchangers	**Location**	**Example**
Plasma membrane Ca^{2+} ATP-ases	Plasma membrane	PMCA1-4
Sodium/Ca^{2+} exchangers	Plasma membrane	NCX1-3
Sarco-endoplasmic reticulum ATP-ases	Endoplasmic Reticulum	SERCA1-3
Mitochondrial channels and exchangers	Mitochondria	Uniporter, NCX
Golgi pumps	Golgi apparatus	SPCA1, SPCA2

Table 4: Off mechanism channels.

In conclusion, the Ca^{2+} toolkit contains a wide range of channels and pumps that allow transient Ca^{2+} to enter into the cytoplasm from the extracellular space and intracellular stores. The duration, localization, and amplitude of the Ca^{2+} increase determine the transduction of the signal. In Figure 1, the main players of the Ca^{2+} toolkit are visualized.

The articles included in this thesis focus on VOCs and $InsP_3R$, and these channels will be described in the next section.

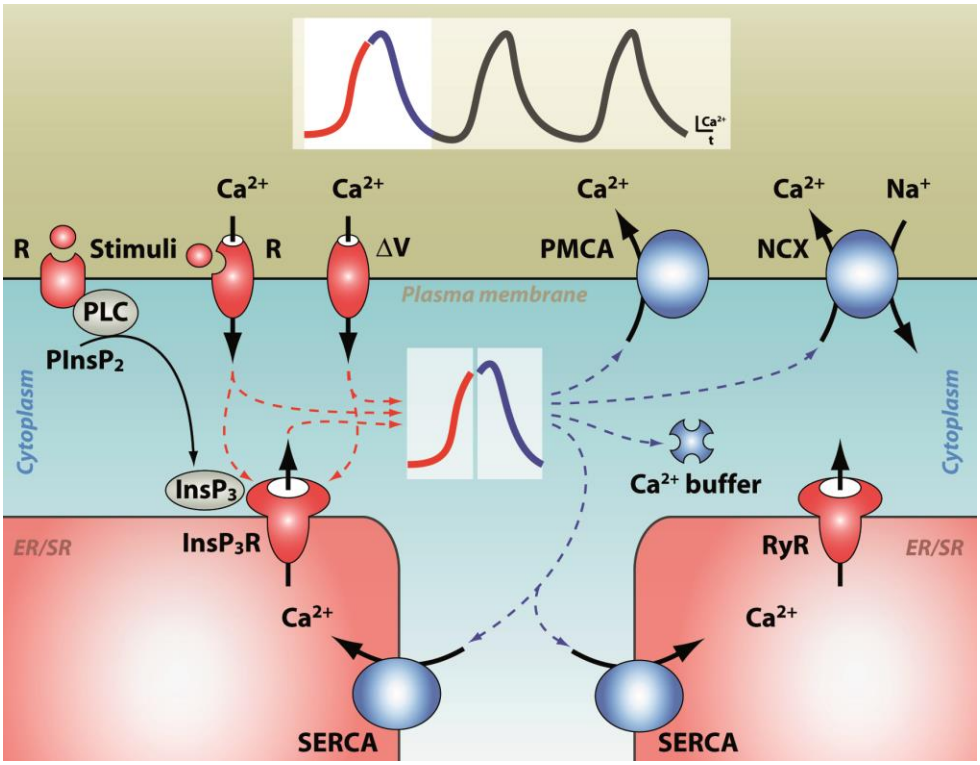

Figure 1: The Ca^{2+} signaling toolkit. The ON mechanisms are shown in red and the OFF mechanisms are shown in blue. Picture from Per Uhlén.

1.1.2 Voltage-operated Ca^{2+} channels and Inositol 1,4,5-trisphosphate receptors

1.1.2.1 Voltage-operated Ca^{2+} channels

Voltage-operated Ca^{2+} channels (VOCs) are fundamental transducers of changes in membrane potential into intracellular Ca^{2+} transients initiating physiological events. They are characterized by activation and inactivation periods. Depending on their physiological and pharmacological properties, voltage-dependent Ca^{2+} channels are divided into high voltage-activated (HVA) and low voltage-activated (LVA) channels. HVA Ca^{2+} channels are comprised of five different subunits (α1, $\alpha_2\delta$, β, and γ), while LVA channels are comprised of only an α1 subunit. The α1 subunit is responsible for the properties of the channels, so different voltage-dependent Ca^{2+} channels are usually referred to by their α1 subunits. For example, there are four different α1 subunits for L-type Ca^{2+} channels: 1S, 1C, 1D, and 1F (table 5). In total, there are ten members of the voltage-operated Ca^{2+} channel family in mammals, and they play distinct roles in cellular signal transduction. The Ca_v1 (L-type) subfamily, a

HVA Ca^{2+} channel that is sensitive to dihydropyridines, initiates contraction, secretion, regulation of gene expression, integration of synaptic input in neurons, and synaptic transmission. The Ca_v2 (N, P/Q and R-type) subfamily is primarily responsible for the initiation of synaptic transmission at fast synapses. The Ca_v3 (T-type) subfamily is important for repetitive firing of action potentials in rhythmically firing cells, such as cardiac myocytes and thalamic neurons (Catterall, 2011). Of these, T-type channels are the first Ca^{2+} channels to be expressed in developing neurons (Chemin et al., 2002).

(Tsien et al., 1988)	(Snutch et al., 1990)	(Ertel et al., 2000)	Voltage type	Associated subunits
L-type ("Long Lasting" or "DHP Receptor")	α 1S α 1C α 1D α 1F	$Ca_v1.1$ $Ca_v1.2$ $Ca_v1.3$ $Ca_v1.4$	HVA	$\alpha_2\delta,\beta,\gamma$
P-type ("Purkinje")/ Q type Ca^{2+} channel	α 1A	$Ca_v2.1$	HVA	$\alpha_2\delta,\beta,(\gamma)$
N-type ("Neural" or "Non-L")	α 1B	$Ca_v2.2$	HVA	$\alpha_2\delta/\beta_1$, β_3, $\beta_4,(\gamma)$
R-type ("Residual")	α 1E	$Ca_v2.3$	HVA	$\alpha_2\delta,\beta,(\gamma)$
T-type ("Tiny" or "Transient")	α 1G α 1H α 1I	$Ca_v3.1$ $Ca_v3.2$ $Ca_v3.3$	LVA	

Table 5: Summary of the different nomenclatures and the associated subunits of the ten types of VOCs.

Comparison of the amino acid sequences of the individual calcium channels revealed relationships among the channel classes. An early evolutionary event separated the α1 subunits into LVA and HVA channels and a later evolutionary event divided the HVA channels into two subfamilies, L-type and neuronal types. Individual members of both subfamilies share greater than 80% sequence homology (Figure 2).

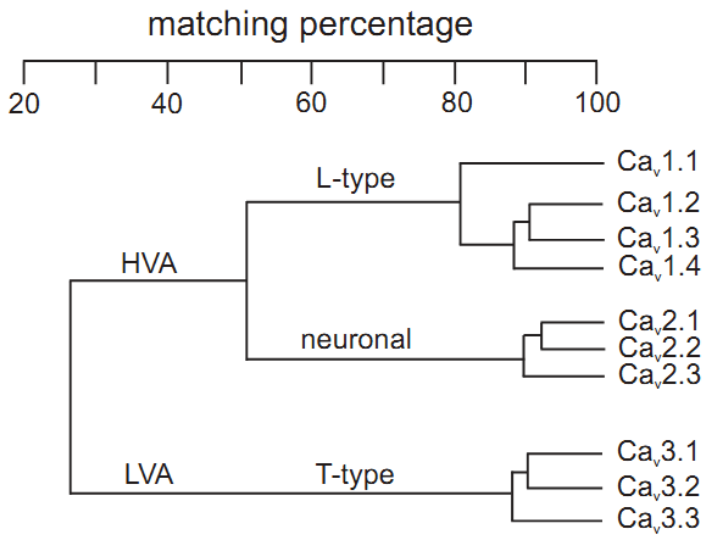

Figure 2: Homology among the different VOCs (Lacinova, 2005).

1.1.2.2 Inositol 1,4,5-trisphosphate receptor

The $InsP_3R$, a tetramer located on the ER membrane, is the mediator of the cellular response to $InsP_3$ (Streb et al., 1983), and is present as three different subtypes, $InsP_3R1–R3$. All three isoforms allow the release of Ca^{2+} and are expressed in most cells, but their different mechanistic and molecular properties mediate different physiological events (Mendes et al., 2005; Wagner and Yule, 2012). $InsP_3R1$ is highly expressed in the Purkinje cells in the cerebellum and the Ca1 pyramidal cell layer of the hippocampus. Knockout animals for $InsP_3R1$ exhibit ataxia and epileptic seizures and die prematurely (Matsumoto et al., 1996). The primary phenotypes exhibited by $InsP_3R2–R3$ double knockout mice are impaired saliva secretion and growth abnormalities (Futatsugi et al., 2005).

There are five functional domains in the receptor: an N-terminal coupling/suppressor domain, an $InsP_3$-binding core domain, an internal coupling domain, a transmembrane/channel-forming domain, and a gatekeeper domain (Mikoshiba, 2007). The domain responsible for the difference in the affinity of $InsP_3$ for different $InsP_3R$ subtypes is the suppressor domain (Iwai et al., 2007).

Activation of $InsP_3R$ by the $InsP_3$ molecule stimulates Ca^{2+} diffusion from the ER through the receptor. $InsP_3$ is formed by the hydrolysis of $PInsP_2$ by activated phospholipase C (PLC), accompanied by the release of diacylglycerol (DAG) (Figure 3). Ca^{2+} and $InsP_3$ also act as co-activators of $InsP_3R$ in a concentration and isoform-dependent manner. For example, the opening of $InsP_3R$ is inhibited both in

high and low Ca^{2+} conditions and so only moderate increases in cytosolic Ca^{2+} can open the channel (Bezprozvanny et al., 1991; Choe and Ehrlich, 2006; Tu et al., 2005).

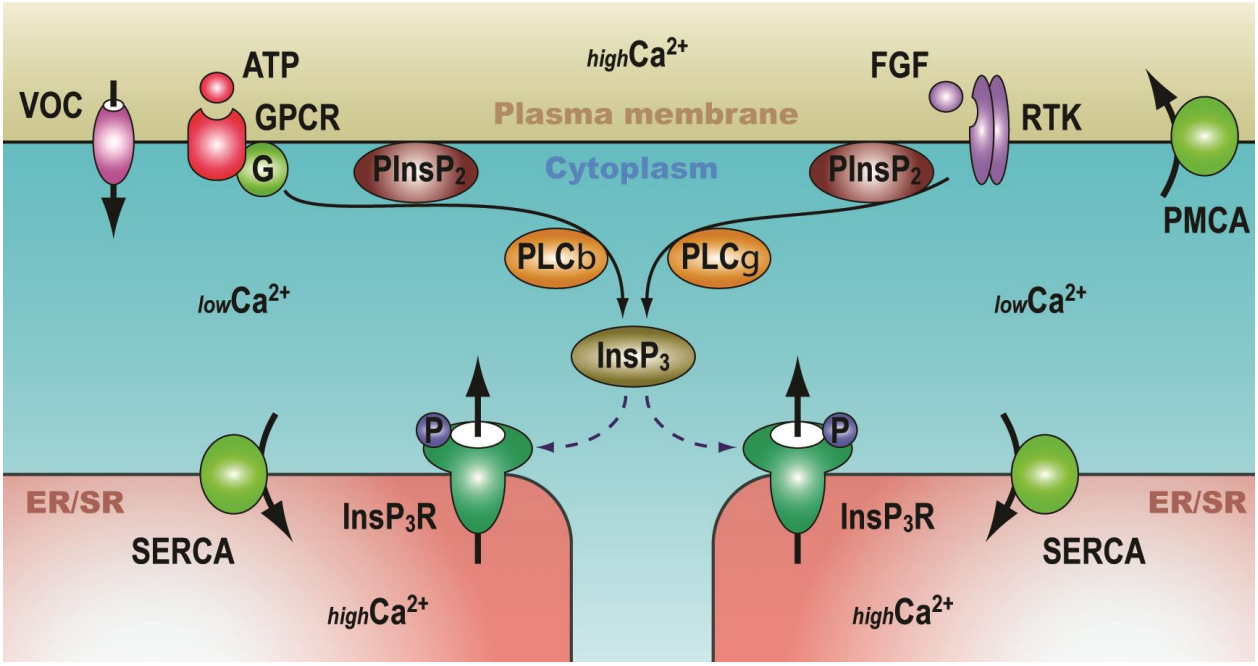

Figure 3: Pathway of $InsP_3$ and activation of $InsP_3R$. Figure from Per Uhlén.

1.1.3 Types of Ca^{2+}signals

An elementary event in calcium signaling is an increase in intracellular Ca^{2+} close to the channel that allows the Ca^{2+} to diffuse from the extracellular space or the intracellular stores. This event is the primary component of Ca^{2+} signaling and has different names and characteristics depending on its origin: Puffs, Bump, and BOB are produced by the $InsP_3Rs$; RyRs generate Sparks, STOC, and SMOC; and VOCs give rise to QED (Bootman and Berridge, 1995). Elementary elements can regulate many localized cellular processes or combine to produce larger signaling microdomains, which are especially important in cardiac and muscular physiology (Wang et al., 2004).

The most common forms of Ca^{2+} signals are transients, oscillations and sustained signals (Uhlen and Fritz, 2010). Ca^{2+} oscillations are comprised of multiple Ca^{2+} transients (peaks) and can be induced by various compounds, such as hemolysine (Uhlen et al., 2000), ouabain (Aizman et al., 2001), and testosterone (Estrada et al., 2006). Ca^{2+} oscillations have been implicated in the control of numerous

biological processes, including oocyte activation at fertilization (Miyazaki et al., 1993), proliferation of neural progenitors (Weissman et al., 2004), differentiation (Ciccolini et al., 2003), and the establishment of neurotransmitter cell phenotypes (Borodinsky et al., 2004).

As the Ca^{2+} ion cannot undergo modification, changes Ca^{2+} in concentration must be very flexible in space, time, and form, but also precisely regulated to coordinate all Ca^{2+} functions.

1.1.3.1 Spatial range

Transient oscillations and sustained signals occur in the cytoplasm and have a wide spatial range. Ca^{2+} signals can be localized, diffusing only nanometers, as in the case of sparks and puffs, but can also be very large, covering even centimeter distances in waves (Bootman et al., 1997). The first Ca^{2+} signal close to the mouth of the intracellular or PM channels can transmit the signal to an enzyme in the immediate vicinity or recruit additional Ca^{2+} channels, triggering a chain of autocatalytic Ca^{2+} releasing events that give rise to a Ca^{2+} wave.

1.1.3.2 Temporal range

The temporal range is fundamental in Ca^{2+} signaling, varying from microseconds (as in exocytosis), minutes or hours (in proliferation, differentiation, gene transcription), or months (as in memory function). The frequency of the signal determines the effectors that will be activated and thus the physiological output. For example, low frequency Ca^{2+} signals activate NF-kB, and high frequency Ca^{2+} signals activate NFAT (Dolmetsch et al., 1998). Furthermore, CaM Kinase II is able to recognize the frequency of the oscillations and vary its activity accordingly (De Koninck and Schulman, 1998; Wheeler et al., 2008).

1.1.3.3 Amplitude

The amplitude of Ca^{2+} signals can be measured, but it is technically challenging because most of the dyes used in Ca^{2+} imaging are also Ca^{2+} buffers. Some genes can be activated by varying the amplitude of Ca^{2+} signals in relation to frequency and duration (Berridge et al., 1998; Dolmetsch et al., 1997; Li et al., 2012).

1.1.4 Cellular consequences

Ca^{2+} ions were discovered to be fundamental for many physiological processes, including fertilization, differentiation, exocytosis, gene expression/transcription, memory function, proliferation, and cell death. This explains the most famous quotation regarding Ca^{2+}, credited to Otto Loewi (1873-1961), a Nobel-prize winning physiologist and professor at New York University:

"*Ja, Kalzium, das ist alles*"
(Yes, *Ca^{2+}* is everything)

Here, a brief summary of the main Ca^{2+} related physiological consequences is presented. Proliferation, differentiation, and migration will be examined in depth in the following sections.

1.1.4.1 Fertilization

It has been known from the early 1920s that eggs can be activated by raising their free Ca^{2+} concentration (Loeb, 1921), which depolarizes the PM (Jaffe, 1985). This happens after the introduction of cationic channels into the PM of the eggs by the sperm (Lynn and Chambers, 1984), provoking activation of PLCζ and the release of Ca^{2+} from internal stores. Consequent Ca^{2+} induced Ca^{2+} release (CICR) sustains the oscillation for some hours (Miyazaki et al., 1992). Recently, it has been demonstrated that Ca^{2+} influx from the extracellular space is fundamental for replenishing Ca^{2+} stores and for the activation of signaling pathways upstream of CaMKIIγ that are required for complete egg activation (Miao et al., 2012).

1.1.4.2 Proliferation

Ca^{2+} has a fundamental role in the mammalian cell cycle and is especially important early in G1, at the G1/S and G2/M transitions (Kapur et al., 2007; Roderick and Cook, 2008). The role of Ca^{2+} in proliferation was well studied in lymphocyte activation, where antigen binding activates PLCγ and $InsP_3$. Ca^{2+} recruited from the ER then activates store-operated Ca^{2+} channels (SOCs), stimulating progression of the cell cycle through store-operated Ca^{2+} entry (SOCE) (Feske, 2007).

1.1.4.3 Differentiation

Ca^{2+} has been shown to regulate many aspects of differentiation, from the induction of a cell phenotype to the development of cell-specific features. In neurons, for example, spontaneous Ca^{2+} events have been correlated to dendrite outgrowth and neurotransmitter phenotypes (Borodinsky et al., 2004; Ciccolini et al., 2003).

1.1.4.4 Migration

Migration of mature neurons towards their final destination is also Ca^{2+} dependent, and treatment with N-type Ca^{2+} channel inhibitors decreases neuronal migration (Komuro and Rakic, 1996). Recently, T-type Ca^{2+} channels have also been reported to affect migration in cultured neurons (Louhivuori et al., 2013), and intracellular Ca^{2+} stores have been shown to play a role in neuronal migration as well (Guan et al., 2007; Pregno et al., 2011).

1.1.4.5 Gene transcription

Since the 1980s, changes in intracellular Ca^{2+} fluxes have been known to affect gene transcription. The first observation of this was made during a study of the expression of the prolactin gene in culture of CH_3 cells (White, 1985). Since then, the number of genes reported to be Ca^{2+} regulated has increased rapidly. Both late response and immediate-early genes, including the c-fos proto-oncogene, have been determined to be Ca^{2+} sensitive. Ca^{2+} sensitive genes are able to discriminate between fluxes through the voltage Ca^{2+} channels and glutamate ion channels in neurons because of their different localization in the PM, i.e., dendrites versus soma (Bading et al., 1993). Most of the information on the role of Ca^{2+} in gene transcription stems from work on CREB, a transcription factor that binds to the cAMP responsive element (CRE) and the Ca^{2+} response element (CARE) and is activated upon CaMKIV activation. The activation of genes by Ca^{2+} oscillations is more effective than that by sustained Ca^{2+} increase, since prolonged Ca^{2+} increase can become toxic for cells (Carafoli et al., 2001). Ca^{2+} mediated CREB activity is an example of when an effector (in this case, the Ca^{2+} dependent phosphatase calcineurin) is able to decode information in the temporal aspect of Ca^{2+} into functionally specific signals (Schwaninger et al., 1995). Another transcription factor activated after dephosphorylation by calcineurin is NFAT (Clipstone and Crabtree, 1992). Ca^{2+} can also directly affect gene transcription, through the downstream regulatory element antagonist modulator (DREAM) in the prodynorfin gene (Carrion et al., 1999).

1.1.4.6 Memory and Learning

Processes that change the strength of synapses, such as long-term potentiation (LTP) and long-term depression (LTD), are generally assumed to underlie memory storage. The α isoform of CaMKII is the most abundant protein in the postsynaptic density (Kennedy, 1993) and can affect the storage process via structural modification of proteins.

1.1.4.7 Cell death

A typical aspect of many pathological conditions is the excessive entry of Ca^{2+} through the PM, termed Ca^{2+} overload.

Ca^{2+} overload leads to the permanent activation of signaling pathways, including those that mediate the activation of hydrolytic enzymes (proteases). Cells attempt to cope with the cytosolic increase in Ca^{2+} by activating removal systems, particularly via the mitochondria. Ca^{2+}-dependent mitochondrial activation is depicted in Figure 4. During conditions of acute cellular stress, mitochondria can store large amounts of Ca^{2+} in the form of hydroxyapatite granules. However, if the Ca^{2+} overload conditions persist, a point of no return will be reached when mitochondria lose their membrane potential and the ability to produce ATP, thus depriving energy to the pumps that remove the calcium. As a consequence, the cell will undergo death through necrosis (Fleckenstein et al., 1974).

Apoptosis is another form of cell death that is not linked to pathological conditions. Apoptosis is a normally programmed event during development and the normal turnover of adult cells. The classic apoptotic proteins, caspases, are not directly Ca^{2+} dependent, unlike the Ca^{2+}-activated thiol protease calpain (Guroff, 1964).

Necrosis, on the other hand, has long been considered an unregulated process. However, recent evidence suggests that necrosis can also take place in a controlled procedure called necroptosis (Vanlangenakker et al., 2008).

Recent findings suggest that the process through which cell death occurs is not pre-determined, but can be decided based on the severity of the injury and the status of the cells, specifically the resting concentration of ATP and the mitochondrial status (Orrenius et al., 2003).

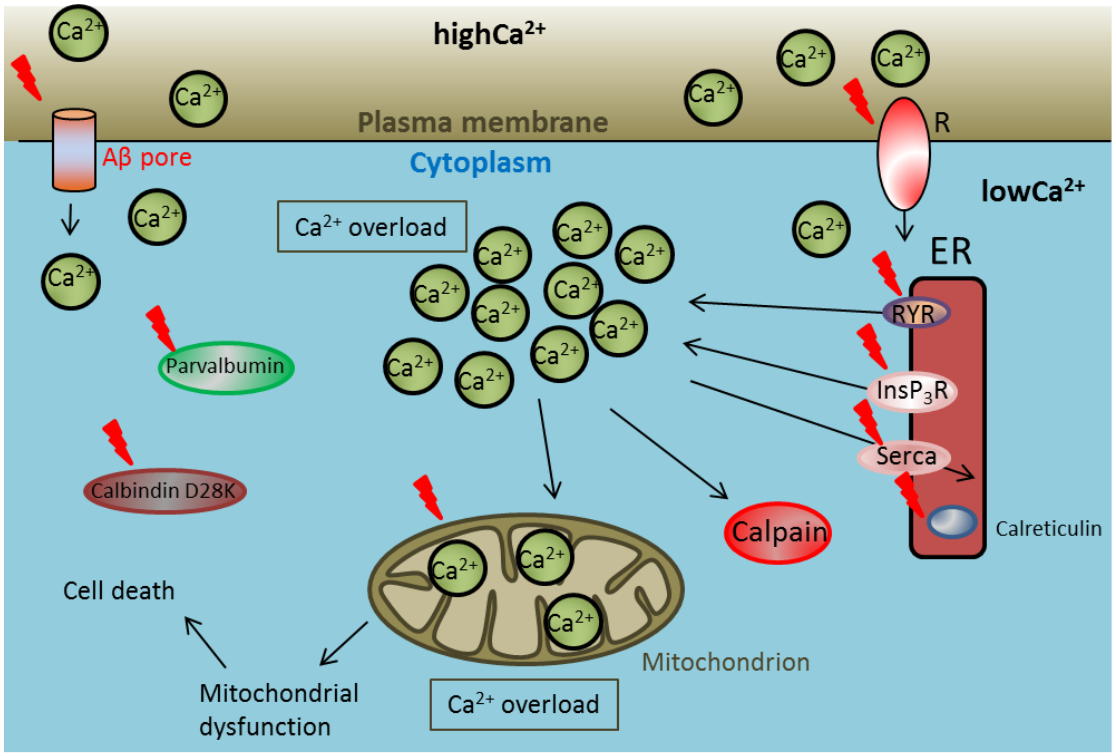

Figure 4: Causes and consequences of Ca^{2+} overload in neurons. Ca^{2+} channels, Ca^{2+} buffering proteins and mitochondrial functions are deregulated in pathological conditions. Figure modified from (Marambaud et al., 2009).

1.1.4.8 Secretion

Secretion of active compounds in intracellular vesicles occurs frequently in response to cellular stimulation. This event is mediated by second messengers, among whom Ca^{2+} is particularly important. The first to identify Ca^{2+} as playing a role in this process were Hodgkin and Keynes (Hodgkin and Keynes, 1957), who suggested that influx into the nerve terminals could have a role in the secretion of acetylcholine. Nowadays, it is well known that the basis of the process is the fusion of the vesicles that store the compound to be secreted with the PM. Moreover, exocytotic emission of the compound into the extracellular space occurs in a Ca^{2+} dependent manner. Within the same cells, different granule populations may be secreted with different Ca^{2+} affinities (Nusse et al., 1998) depending on the involvement of different Ca^{2+} sensors, such as synaptotagmin, annexins, S-100 proteins, and calmodulin (Sudhof and Rizo, 1996).

1.1.4.9 Contraction

In the 1950s, Sandow proposed that Ca^{2+} could link action potential in the PM to myosin contraction (Sandow, 1950). Later, it was shown that activating Ca^{2+} came from intracellular stores located in the terminal cisternae of the sarcoplasmic reticulum.

Contraction of skeletal and cardiac muscles was later shown to be mediated not by myosin, but rather by a set of proteins that includes actin, tropomyosin, and troponin. Troponin C, one of the proteins in the troponin complex, was demonstrated to be the Ca^{2+} sensor in the myofibrils via its EF-hands motifs (Hitchcock, 1975). In smooth muscles, action potential brings in sufficient Ca^{2+} to activate contraction through a Ca^{2+}-calmodulin dependent process (Carafoli et al., 2001; Sparrow et al., 1981).

1.1.4.10 Regulation of enzymes

Ca^{2+} indirectly regulates phosphorylation and dephosphorylation on the serine/threonine residues of many enzymes, usually after interaction with and activation of CaM. Example of kinases and phosphatases that are regulated by Ca^{2+} are listed in Table 3.

1.2 Ca^{2+} SIGNALING AND PROLIFERATION

1.2.1 Ca^{2+} dependent proliferation

As reported in the previous section, Ca^{2+} signaling is important for the proliferation and regulation of the cell cycle, mainly in G1 and at the G1/S and G2/M transitions. Both increases in the basal cytosolic calcium concentrations ($[Ca^{2+}]_i$) and $[Ca^{2+}]_i$ transients play a major role in cell cycle progression, cell proliferation, and division.

How is this mediated? Cell cycle and cell division are under the strict control of cyclins and CDK complexes. Ca^{2+} regulates the expression, activity, and localization of the transcription factors that control G1 cyclins (Fos, Jun, MyC, CREB–ATF1, and NFAT), but also acts directly on the cyclins after stimulation of CaM (Kahl and Means, 2003), as shown in Figure 5. Ca^{2+} and CaMKII also control centrosome duplication and separation, allowing the distribution of replicated chromosomes to daughter cells. Furthermore, Ca^{2+} oscillations occur at the G1/S boundary (centrosome duplication) and the G2/M transition (centrosome separation), during which CaMKII localizes to the centrosomes (Roderick and Cook, 2008).

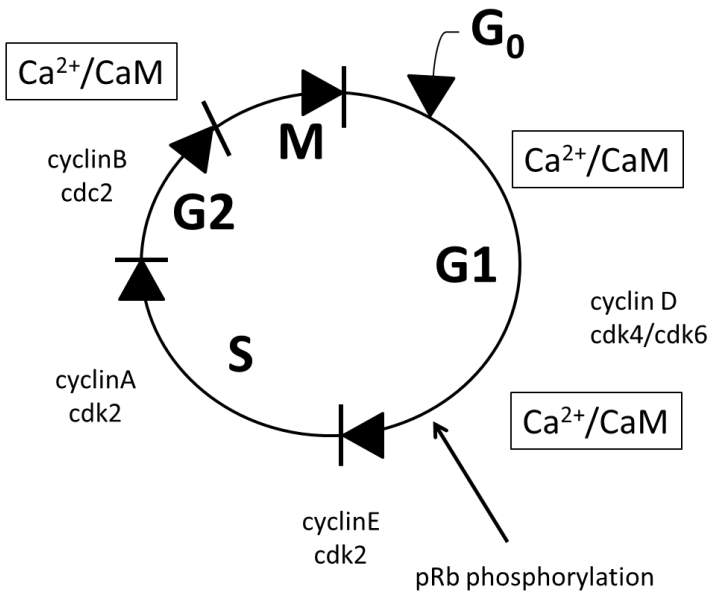

Figure 5: Ca^{2+} dependent regulation of cell cycle. Ca^{2+} /CaM is required at two points during the reentry from quiescence, early after mitogenic stimulation and later near the G1/S boundary.

Additionally, Ca^{2+}/CaM is implicated in the G2/M transition, M phase progression, and exit from mitosis. Figure modified from (Kahl and Means, 2003).

1.2.2 Ca^{2+} channels affecting proliferation

The use of Ca^{2+} channel blockers has been one of the main arguments in support of the role of calcium in cell proliferation. Drugs that block L- and T-type VOCs and SOCE and NCCE channels, such as verapamil, diltiazem, mibefradil, 2-APB, SK&F 96365, and carboxyamidotriazole, have been shown to have anti-proliferative effects in several tissues (Chung et al., 1994; Enfissi et al., 2004; Panner and Wurster, 2006; Taylor and Simpson, 1992).

L-type Ca^{2+} channels have been connected to neural proliferation in cells from rat mesencephalon kept under hypoxic conditions (Guo et al., 2010), but the underlying mechanism of this effect is still unknown.

T-type calcium channels are widely expressed in cancer cells. The unique low voltage-dependent activation/inactivation and slow deactivation of T-type calcium channels indicate that they may carry a depolarizing current at low membrane potentials. These channels could play an important role in regulating Ca^{2+} in non-excitable tissues. At low voltage, T-type calcium channels mediate a mechanism called “window current” (Crunelli et al., 2005) caused by a voltage overlap between activation and steady state inactivation. This results in a sustained inward calcium current carried by a small portion of channels, regulating calcium homeostasis at low or resting potential (Bean and McDonough, 1998).

A complex sequence of events involving Orai1 and Orai3 in the PM and STIM1 and STIM2 on the ER membrane is necessary for SOCE activation. SOCE is triggered by depletion of the Ca^{2+} stores of the ER through InsP3 or RyRs and subsequent refilling of the intracellular stores by Ca^{2+} entry through PM channels. This can give rise to Ca^{2+} oscillations that have been implicated in cell cycle progression in mouse embryonic stem (ES) cells (Kapur et al., 2007; Varnai et al., 2009). STIM1 also binds to transient receptor potential canonical cationic channels (TRPCs), suggesting a role for TRP channels in SOCE (Capiod, 2011; Zitt et al., 2002).

Another set of PM calcium channels can be activated independent of ER Ca^{2+} depletion and calcium entry via other second messengers, such as DAG (Gudermann et al., 2004). This type of calcium entry is known as non-capacitative calcium entry (NCCE) and is involved in the proliferation of non-excitable cells (Capiod, 2013).

1.2.3 Ca^{2+} and gap junctions in neural proliferation

Gap junctions are channels that form a connection between the cytoplasm of two adjacent cells and allow the exchange of electrical currents and small molecules (<1 kDa). Two hemichannels on opposing membranes make up one gap junction. Hemichannels are composed of six connexin (Cx) subunits, each having four transmembrane domains and two extracellular loops (Evans and Martin, 2002).

Gap junctions were first described in the mature brain in the late 1970s. There are 20 genes encoding different connexins in rodents and humans with distinct permeability and regulation properties. Five of these are highly expressed in the rodent embryonic cerebral cortex, including Cx26, Cx36, Cx37, Cx43, and Cx45, with a distinct spatial and temporal pattern that gives rise to significant functional differences. Cx26, Cx37, and Cx45 are largely distributed from the ventricular zone (VZ), the major proliferative area of the developing cortex, to the cortical plate, whereas Cx36 and Cx43 are highly expressed in the VZ and less in the cortical plate (Cina et al., 2007; Nadarajah et al., 1997).

Gap junctions have been recently shown to govern many different aspects of development, including coupling, hemichannel function, adhesion, and signaling. Systematic intercellular contacts also mediated by gap junctions determine the complexity of the cerebral cortex. Indeed, the ability of gap junctions to create morphogenic gradients and synchronize electrical activities is fundamental for controlling embryonic morphogenesis and generating cortical circuits, but also as an architectural tool (Elias and Kriegstein, 2008; Elias et al., 2007; Zsiros and Maccaferri, 2008). Moreover, Ca^{2+} waves mediated by gap junctions divide the mammalian neocortex into distinct neuronal domains (Bennett and Zukin, 2004; Yuste et al., 1995). Spontaneous gap junction-dependent Ca^{2+} waves are also observed in the developing retina (Kandler and Katz, 1998), and a novel model in which Ca^{2+} plays dual roles in directing the fate of a specific type of olfactory neuron within the innexin (i.e.,

analog of connexins in invertebrates) network in *C. elegans* was recently proposed (Schumacher et al., 2012).

Ca^{2+} waves propagate through radial glial cells in the proliferative cortical ventricular zone (VZ). Radial glial Ca^{2+} waves require connexin hemichannels, P2Y1 ATP receptors, and intracellular $InsP_3$-mediated Ca^{2+} release. Wave disruption in neural progenitors decreases VZ proliferation during the peak of embryonic neurogenesis (Weissman et al., 2004).

1.3 Ca^{2+} SIGNALING AND DIFFERENTIATION

1.3.1 Differentiation of embryonic stem cell and neuroepithelial stem cells into neurons

ES cells are self-renewing pluripotent cells from the inner mass of the blastocyst that give rise to cells of all three germ layers: endoderm, ectoderm, and mesoderm. Neuroepithelial stem (NS) cells are a population of self-renewing and multipotent cells that can generate the main cell types in the nervous system: neurons, astrocytes, and oligodendrocytes.

The *in vitro* generation of neurons from ES and NS cells is a promising approach for producing neurons for cell-based replacement therapies of the nervous system as well as developmental studies. The challenge is to try to replicate the complex process of embryonic development in a reproducible and efficient way using all the available methods. To do so, a deep understanding of the cellular and molecular events that are involved in this process is required.

Many different approaches have been used to achieve *in vitro* neural differentiation, focused primarily on generating regionally specific neural progenitors or differentiated neuronal and glial subtypes. Initially, the most common methods were embryoid body (EB) formation in the presence of retinoic acid (Bain et al., 1995) or co-culture of ES cells with stroma/conditioned medium (Kawasaki et al., 2000). However, as ES cells are pluripotent and thus have the capacity to differentiate into almost any cell type, the efficiency of neural conversion was limited and lineage selection was usually necessary to ensure the homogeneity of the differentiated population (Li et al., 1998).

A simpler way to reconstitute neural commitment *in vitro* and achieve efficient neuronal production relies upon monolayer differentiation of ES cells, a method developed by Ying and co-workers (Ying et al., 2003) in which ES cells are cultured in defined serum- and feeder-free conditions in the absence of BMP signals, which are known to inhibit neural fate. Under these conditions, ES cells undergo neural commitment through an autocrine induction mechanism, in which FGF signaling plays a crucial role, just as it does in the embryo (Stavridis et al., 2007). This method results in a

more efficient neural commitment and differentiation, which likely results from a more authentic mimicry of the events that occur in the embryo, especially in cortical development.

Neuroepithelial progenitors cells derived in medium supplemented with N2B27 organize into neural tube-like rosettes where they display the morphological and functional characteristics of their embryonic counterparts, namely, apico-basal polarity, active Notch signaling, and proper timing of production of neurons and glia (Abranches et al., 2009). Such spontaneous organization has been shown in both mouse and human differentiating ES cells (Gaspard et al., 2009; Shi et al., 2012).

1.3.2 Ca^{2+} dependent neural induction

Spontaneous Ca^{2+} events appear to be common occurrences in the developing brain. In Zebrafish and amphibian embryos, localized Ca^{2+} transients have been imaged during gastrulation in the dorsal region. These Ca^{2+} transients were temporally and spatially correlated with neural induction (Leclerc et al., 1997).

In mammals, neural induction studies have mainly involved the use of ES cells due to difficulties in manipulating early embryos. The results obtained from Xenopus and mouse models reveal that the mechanisms that govern neural induction involve cross-talk between several signaling pathways and require inhibition of the BMP pathway, activation of the FGF/Erk pathway, and controlled Ca^{2+} homeostasis. In mouse ES cells, Ca^{2+} signaling increases the phosphorylation of Erk and triggers neural induction (Lin et al., 2010), so an increase in $[Ca^{2+}]_i$ appears to be crucial for the control of neural fate determination in vertebrates.

This increase in $[Ca^{2+}]_i$ may result from an influx of Ca^{2+} through Ca^{2+} channels on the PM and/or Ca^{2+} release from the ER. However, the route of Ca^{2+} increase seems to differ between the amphibian and the mammal models, being dependent on L-type VOCs in Xenopus and TRP channels in mouse, since ES cells do not express VOCs, only TRPC1 and TRPC2 (Leclerc et al., 2012) as shown in Figure 6.

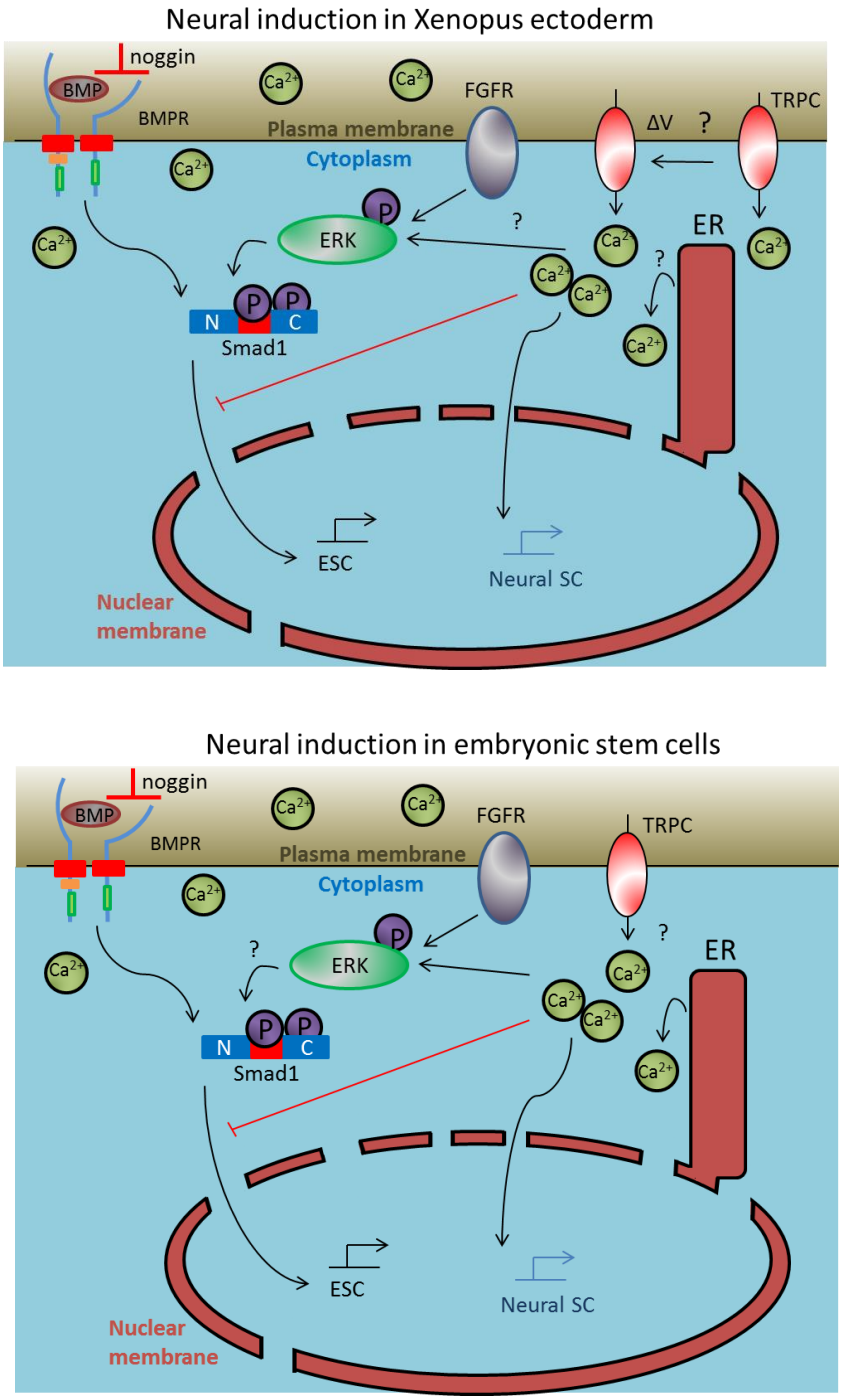

Figure 6: Signaling pathway occurring during neuronal induction in amphibian ectoderm cells and in ES cells. An increase in intracellular Ca^{2+} concentration is a common signal that drives embryonic cells toward the neural fate. In amphibian, the main source of Ca^{2+} increase rely on an influx through VOCs but ESCs instead do not express VOCs. Both cell types expressed TRP channels, probably TRPC, which could contribute to the Ca^{2+} signals. Figure modified from (Leclerc et al., 2011).

Ca^{2+} release from the ER, the main source of Ca^{2+} in ES cells, is mediated by $InsP_3Rs$ but not by ryanodine receptors (RyRs). Both plasma membrane Ca^{2+}-ATPase (PMCA) and the Na^{+}/Ca^{2+} exchanger (NCX) contribute to the extrusion of Ca^{2+} from the cytoplasm (Yanagida et al., 2004).

1.3.3 Ca^{2+} dependent dendritic outgrowth

The growth, branching, and guidance of neural projections during development are controlled by complex mechanisms that include both diffusible and local Ca^{2+} signals. Spontaneous Ca^{2+} activity occurs during a period of intense dendritic growth in neurons, in which the increase in Ca^{2+} confers stability in the branching of embryonic retinal ganglion cells (Yamashita, 2008).

Calcium signal propagation to the nucleus requires calcium influx primarily through NMDA type glutamate receptors and L-type voltage sensitive calcium channels. Synaptic transmission that contributes to the elevation of intracellular Ca^{2+} levels through VOCs also induces CICR from the intracellular stores and contributes to stabilization of the new branches (Lohmann et al., 2002).

Intracellular Ca^{2+} elevation can affect dendritic growth via downstream regulators, especially through CaMKs activated by the complex calcium/CaM. CaMKII is highly expressed in the brain, and the β isoform of CaMKII is required to initiate branching of dendrites in sympathetic and hippocampal neurons (Fink et al., 2003; Vaillant et al., 2002). The α isoform of CaMKII is required for dendritic growth in cortical neurons (Wu and Cline, 1998).

CaMKIV, which is generally localized in the nucleus, is also involved in dendritic growth in cortical neurons through phosphorylating CREB in response to Ca^{2+} influx through VOCs. Surprisingly, however, CREB activation alone through the classic pathway involving cAMP and PKA is not sufficient to promote dendritic growth (Redmond et al., 2002).

Another transcription factor that is important for dendritic growth is the Ca^{2+}-responsive transactivator (CREST). Analysis of CREST knockout mice revealed defects in the dendritic growth of cortical and hippocampal neurons. In addition, cortical neurons from CREST mutant mice showed impaired dendritic growth in response to depolarization (Aizawa et al., 2004).

The mitogen activated protein (MAP) kinase signaling pathway has been implicated in dendritic growth. Activation of this pathway via sustained activation of ERK1/2 is crucial for stabilization of new neurons in the hippocampus (Wu et al., 2001) and cerebellar granule cells (Borodinsky et al., 2003) and for dendritic growth mediated by the Na^{+}/K-ATPase (Desfrere et al., 2009).

These signaling pathways are summarized in figure 7

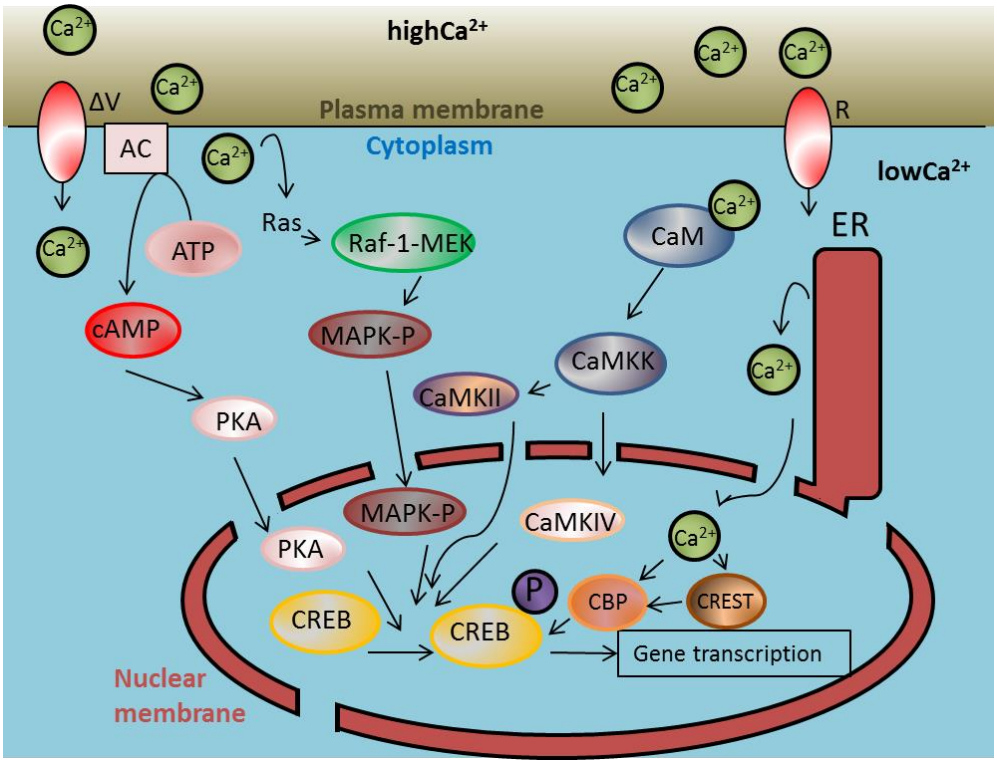

Figure 7: Neuronal Ca^{2+} signaling. Ca^{2+} entry through VOCs or ROCs activates a variety of signaling pathways that regulate gene transcription after phosphorylation of the transcription factor CREB.

1.3.4 Ca^{2+} dependent neurotransmitter specification

The specification of neurotransmitter phenotype has been considered, for many years, a fixed mechanism. However, recent findings have demonstrated that it is dependent on early electrical activity. Molecular or pharmacological alteration of electrical and Ca^{2+} activity can change the number of neurons expressing excitatory and inhibitory transmitters in Xenopus spinal cord in a

homeostatic way. Thus, increasing Ca^{2+} activity increases inhibitory synapses and decreasing Ca^{2+} activity increases excitatory synapses (Figure 8). Changes in transmitter specification are matched by changes in postsynaptic neurotransmitter receptor expression, thus influencing synaptic transmission and affecting behavior (Borodinsky et al., 2004; Spitzer, 2012). Furthermore, a correlation between the GABAergic phenotype and Ca^{2+} activity was shown in differentiating neural stem cells in mice (Ciccolini et al., 2003).

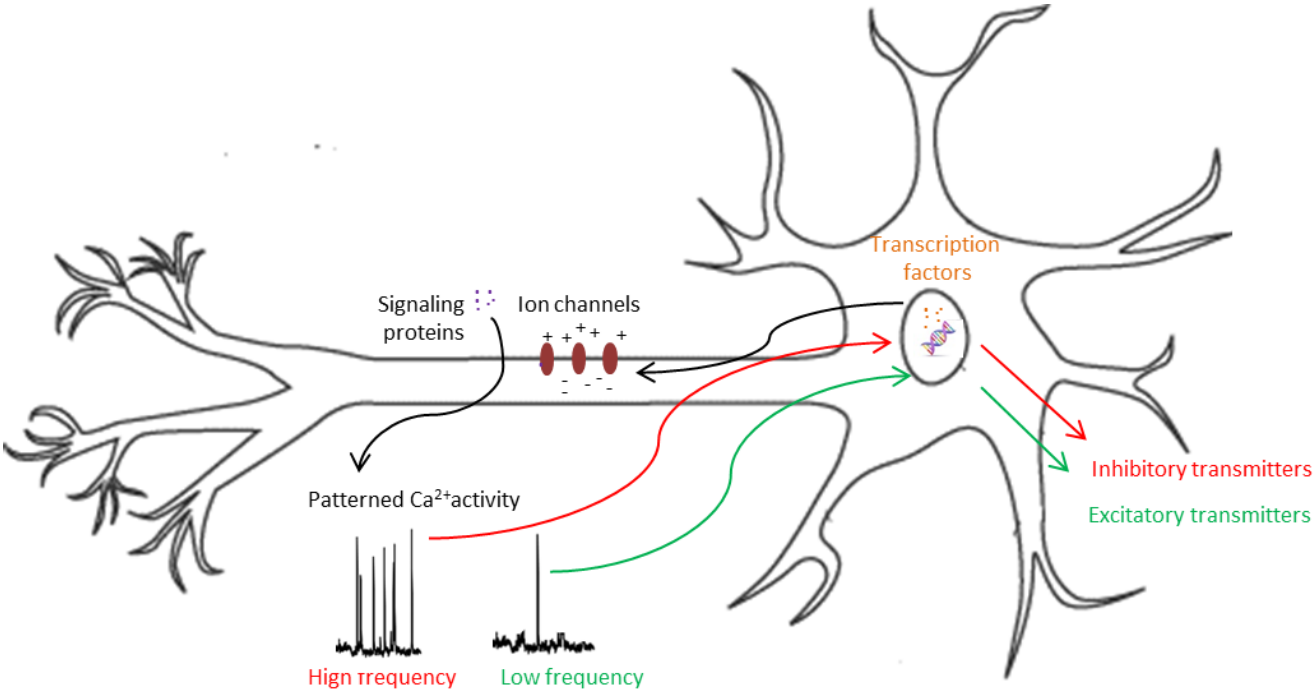

Figure 8: The homeostatic model for neurotransmitters specification. The expression of transcription factors affects the presence of ion channels that produce pattern of Ca^{2+} activity modulated by signaling protein. Different patterns of spike activity activate Ca^{2+} dependent transcription factors and regulate the enzymes that store specific transmitters in a homeostatically way. Figure modified from (Spitzer et al., 2005).

1.3.5 Caspase-3 dependent differentiation

Caspases are **c**ysteine-**asp**artic acid prote**ase**s that have a fundamental role in apoptosis, necrosis, and inflammation. Twelve caspases have been identified in humans, categorized as initiators (caspases 2, 8, 9, and 10) and effectors (caspases 3, 4, 5, 6, 7, 11, and 12). Initiator caspases target other caspases

as substrate, while effector caspases cleave other protein substrates in the cells to trigger apoptosis. This post-translational regulation of caspases assures rapid activation of the apoptotic process (Salvesen and Riedl, 2008).

Caspase-3 is the final executor of the two canonical caspase signaling pathways, the intrinsic (mediated by mitochondria and cytochrome c) and the extrinsic (through death receptor) pathways, as shown in Figure 9. Non-canonical apoptotic pathways are mediated by different caspases.

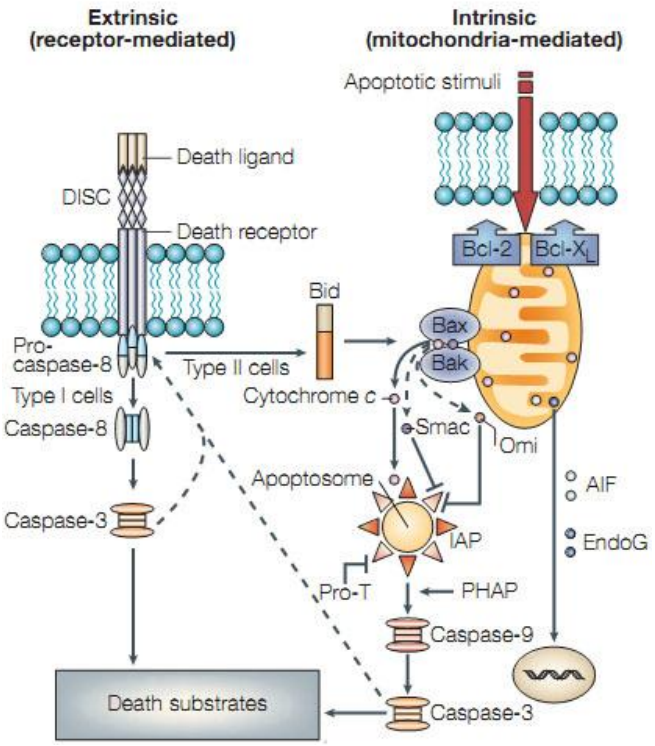

Figure 9: the two different pathways of activation of caspase-3. The extrinsic, receptor-mediated pathway occurs through activation of caspase-8 and the intrinsic mitochondria-mediated pathway requires activation of caspase-9. Figure modified from (Orrenius et al., 2003).

Recently, various non-lethal roles of caspase-3 have been demonstrated in PC12 cells and primary culture of striatal neurons, in which neural differentiation was associated with an increase in caspase-3 activation but not cell death (Fernando et al., 2005; Rohn et al., 2004). Thus, caspase-3 appears to be involved in neurogenesis and synaptic activity (Abdul-Ghani and Megeney, 2008; D'Amelio et al., 2010).

Tissue development and maintenance are dependent on a complex interplay of stem cell self-renewal, differentiation, and apoptosis/programmed cell death. Caspase-induced cleavage of Nanog in differentiating ES cells was demonstrated by Fujita and collaborators, reporting that stem cells lacking the gene coding for caspase-3 showed marked defects in differentiation (Fujita et al., 2008).

The discoveries of new roles for caspases is not limited to stem cells and neurons: recent findings have revealed non-apoptotic roles for caspases in specialized cellular structures, such as immune regulation and spermatogenesis (Yi and Yuan, 2009). Moreover, caspase-3 activity has been associated with the regenerative response after cortical stroke (Fan et al., 2013) and synaptic dysfunctions in the early stages of Alzheimer's disease (D'Amelio et al., 2011).

1.3.6 Perturbation of differentiation: developmental neurotoxicity

Neurotoxicity is defined as the study of the adverse effects induced by exogenous or endogenous factors (biological, chemical, or physical) on the nervous system (Tilson et al., 1995).

The developing central nervous system is continually undergoing remodeling processes in which active proliferation, differentiation, and migration are tightly controlled in time. During development, there is a "window of susceptibility", a period in which the neurotoxic agent in contact with the cells is fundamental for determining the effect on brain maturation. The developing brain is particularly vulnerable to toxic insults compared to the adult brain because of the lack of a functional barrier. The placenta only partially protects the fetal brain and the blood brain barrier is not fully developed until after birth (Adinolfi, 1985). Moreover, fetuses do not possess a complete set of liver enzymes for efficient detoxification of exogenous substances.

Polychlorinated biphenyls (PCBs) and methyl mercury (MeHg) are common food contaminants that raise many concerns because of their persistence and prevalence in the environment. PCBs and MeHg undergo bio-accumulations (i.e., the levels in exposed organisms increase with continued exposure) and bio-magnification (i.e., the levels increase with trophic level).

Understanding the effect of exposure to neurotoxic agents during development is problematic because of the complexity and heterogeneity of the nervous system. *In vitro* models that utilize culture cells originating from the nervous system permit investigation of the molecular mechanism of neurotoxicity. However, exclusion of the effect of the metabolic transformation induced by neurotoxic substances has to be taken in to consideration (Qian et al., 2000).

Because it is so intimately involved in proliferation, differentiation, and cell death, Ca^{2+} signaling can be highly perturbed by the action of neurotoxic agents. Spontaneous Ca^{2+} oscillations are particularly evident during the middle stages of neuronal differentiation (Ciccolini et al., 2003); thus, their frequencies can be used as a marker of proper cell differentiation.

1.4 Ca^{2+} SIGNALING AND NEURONAL MIGRATION

Migration of neuronal precursors and neurons from the site of origin to their final location is a crucial process in the development of the nervous system and the correct organization of neuronal structures and circuits. This aspect of neurogenesis is sequential, but also overlaps with proliferation and differentiation mechanisms and is dependent on Ca^{2+} signaling. Influx of Ca^{2+} from the extracellular medium represents the main mechanism, and a more delimited but specific role is played by Ca^{2+} release from intracellular stores. Moreover, radial and tangential migration in the cerebellum and cortex are governed by different mechanisms, involving VOCs and the neurotransmitters GABA and glutamate, respectively (Lovisolo et al., 2012). Furthermore, early electrical activity dependent on PM Ca^{2+} channels and internal stores affect neuronal migration.

1.4.1 VOC-dependent migration

Many reports have demonstrated the involvement of voltage-dependent Ca^{2+} channels in neuronal migration. In the early 1990s, analysis of neuronal migration in mouse cerebellar slice preparations revealed that postmitotic granule cells initiate migration only after the expression of N-type Ca^{2+} channels. Furthermore, selective blockade of these channels by the addition of ω-conotoxin to the incubation medium decreased cell movement. On the other hand, inhibitors of L- and T-type Ca^{2+} channels, as well as those of sodium and potassium channels, had no effect on the rate of granule cell migration (Komuro and Rakic, 1992). Migration of gonadotropin-releasing hormone-1 (GnRH-1) neurons has also been associated with N-type voltage Ca^{2+} channels (Toba et al., 2005). It has been reported that migrating neurons experience Ca^{2+} oscillations that are dependent on L-type Ca^{2+} channels but that do not affect migration (Darcy and Isaacson, 2009). However, recent observations report a new role for T-type Ca^{2+} channels in neuronal migration. Time-lapse imaging of differentiating neurospheres cultured in the presence of T-type channel blockers showed a significant decrease in the number of actively migrating neuron-like cells and neurite extensions (Louhivuori et al., 2013).

1.4.2 Neurotransmitter-dependent migration

Ca^{2+} signaling also controls neural migration through GABA and glutamate signaling (Platel et al., 2008). It has been shown that the amplitude and frequency of Ca^{2+} oscillations are positively correlated with the rate of granule cell movement in cerebellar microexplant cultures. Moreover, NMDA receptor antagonists reduce neuronal migration in cerebellar slices, whereas activation with glycine or inhibition of glutamate reuptake increases the rate of migration (Komuro and Rakic, 1993, 1996). Recent findings using inhibition and knock-down of TRPC channels delineate a controversial role for these channels in migration (Ariano et al., 2011; Storch et al., 2012).

1.4.3 Internal stores dependent migration

The chelation of intracellular Ca^{2+} with 10 μM BAPTA-AM and decrease of internal Ca^{2+} release with 1 μM thapsigargin results in a significant reduction in Ca^{2+} frequency in the granule cell somata and decreased cell movement. Furthermore, inhibition of upstream Ca^{2+} signaling by inhibition of phospholipase C (PLC) with 1 μM U73122 also significantly decreased Ca^{2+} transient frequency and cell movement (Komuro and Kumada, 2005). Neuregulin1 induces migratory activity through a long-lasting increase in $[Ca^{2+}]_i$ that is dependent on the release of Ca^{2+} from intracellular stores and consequent activation of SOCE (Pregno et al., 2011). CICR is also involved in neuronal migration, since it underlies the long-range Ca^{2+} signaling from the growth cone to the soma that mediates the reversal of neuronal migration induced by slit-2, a repulsive factor (Guan et al., 2007).

2 AIMS

The goal of this thesis was to investigate the different roles of Ca^{2+} in neurogenesis. Specifically, my defined aims were to:

1-Examine and characterize spontaneous Ca^{2+} oscillations and their role in stem cell-derived neural progenitors.

2-Define the role and molecular consequences of T-type channel-dependent Ca^{2+} signaling during the differentiation of human neural progenitors.

3-Describe the effect of non-cytotoxic concentrations of polychlorinated biphenyls (PCBs) on neurogenesis using spontaneous neuronal Ca^{2+} oscillations to monitor the state of the cells.

4-Study the mechanism and role of Ret-dependent Ca^{2+} signaling.

3 RESULTS AND DISCUSSION

3.1 PAPER I: NEURAL PROGENITORS ORGANIZE IN SMALL-WORLD NETWORKS TO PROMOTE CELL PROLIFERATION

3.1.1 Neural progenitors differentiating from mES cells display spontaneous Ca^{2+} activity

In this project, we focused on spontaneous Ca^{2+} activity in neural progenitor cells. Mouse ES cells were plated on PA6 stromal cells and differentiated towards dopaminergic neurons. Cells were loaded with Ca^{2+} sensitive dye and time-lapse recording was performed between day 0 and 14 of differentiation to examine intracellular Ca^{2+} homeostasis in neural progenitors. Spontaneous activity was present from day 7–14 in neural culture conditions, when cells expressed both the neural progenitor marker nestin and the neuronal marker Tuj1. Using *in vivo* imaging, we demonstrated that neural progenitor cells in E9.5 embryos also display spontaneous Ca^{2+} activity.

3.1.2 Cross-correlation and network analysis show that neural progenitor Ca^{2+} signaling is highly coordinated

Cross-correlation analysis, a well-established mathematical method for analyzing multiple time series data, was performed to determine if spontaneous Ca^{2+} activity is independent, due to pacemaker activity, or if cells are connected in clusters. Cross-correlation analysis of undifferentiated ES cells showed low correlation between neighboring cells, while differentiating cells were connected to neighboring cells forming neural networks. The formation and presence of a network was then studied with an algorithm implemented in MATLAB, revealing a distinct group of cells with a high correlation coefficient and low intercellular distance. Network analysis revealed the presence of a scale-free and small-world network. Scale-free properties are suggested by the presence of hubs cells, which were highly connected to other cells. Small-world networks are instead related to the connectivity between most of the cells, meaning that most nodes (cells) can be reached from every other node in a small number of steps (connections). Many real networks have been shown to possess small-world properties; for example, newborn human brains and cultured neurons (Fransson et al., 2011).

3.1.3 Ca^{2+} enters from plasma membrane channels that are dependent on gap junctions to become activated

To identify the source of Ca^{2+} and signaling mechanism in neural progenitors, we challenged the cells with a wide range of pharmacological agents. ATP receptor antagonists (suramin and PPADS) had no effect on spontaneous activity, nor did the extracellular ATP depletion agent, hexokinase. The SERCA pump blockers cyclopiazonic acid and thapsigargin also failed to inhibit activity, and the same was observed for the synaptic transmission blockers tetradotoxin (TTX, a Na^{2+} channel blocker), D-AP5 and NBQX (NMDA and AMPA blockers, respectively). The removal of extracellular Ca^{2+} completely inhibited spontaneous activity, as did low concentrations of nickel (50 µM), which blocks VOCs. Moreover, the gap junction blockers 1-octanol (1 mM) and flufenamic acid (FFA, 100 µM) effectively inhibited most spontaneous Ca^{2+} activity.

3.1.4 *In vitro* and *in vivo* electrophysiological experiments reveal that neural progenitors are electrically connected

Whole cell patch clamp was performed on neural progenitors, revealing high frequency activity that was blocked by 1 mM 1-octanol and 50 µM nickel and 100 µM cadmium. To investigate the interconnectivity between touching and non-touching progenitor cells, multi-electrode patch-clamp recordings were carried out on differentiating neural progenitors and E9.5 embryonic midbrain tissue. The results showed that neural progenitors both *in vivo* and *in vitro* are electrically connected through gap junctions. Moreover, electrical coupling was blocked by 1-octanol and flufenamic acid and 18α-glycyrrhetinic acid, confirming the role of gap junctions.

3.1.5 Gap junction-dependent Ca^{2+} oscillations are fundamental for neural progenitor proliferation

We then analyzed the physiological role of gap junction-related Ca^{2+} signaling mechanisms. The inhibition of Ca^{2+} activity with long-term treatment with 1-octanol resulted in reduced BrdU incorporation and a decrease in the number of proliferating cells, suggesting a role for this signaling mechanism in the expansion of the neural progenitor pool. Intracellular Ca^{2+} signaling has been

implicated in the transition between the G1 and the S phases (Kapur et al., 2007; Roderick and Cook, 2008), and such transition was clearly inhibited by 1-octanol in our system. On the other hand, proliferation assays on undifferentiated ES cells showed no effect of treatment with 1-octanol or nickel.

3.1.6 Connexin 43 is highly expressed in differentiated cells and regulates neural progenitor proliferation

As explained in section 1.2.3, there are several types of gap junctions that are formed by different connexins (Cx). We analyzed the expression profile of the different connexins and found that Cx43 is the most highly expressed throughout the differentiation of mES. To confirm the involvement of Cx43 in proliferation of neural progenitors, we performed knock-down of Cx43, resulting in a lower percentage of proliferating cells compared to control. Conversely, over-expression of Cx43 had no detectable effect on Ca^{2+} activity, networking, or proliferation, most likely due to saturated levels of endogenous Cx43.

3.1.7 *In vivo* analysis of the inhibition of gap junction revealed reduced proliferation of neural progenitors without an increase in the number of apoptotic cells

To examine whether acute pharmacological blockade of gap junctions affects proliferation and brain development *in vivo,* we injected 1-octanol (0.5 mg/g body weight) intraperitoneally into pregnant mice at E12.5. At E17.5 days, EdU was injected and after 1 hour, embryos were removed and immunostained. Treatment of pregnant mice with 1-octanol affected the size of the embryonic brain and the number of neural progenitors in S-phase. Moreover, the thickness of the cortical layers as indicated by the markers satb2, tbr1, and ctip2 was reduced in 1-octanol-treated animals. Brain surface area was also decreased, demonstrating a clear role for gap junction-related Ca^{2+} signaling in neural proliferation. Cx43 knockout mice would have been a useful tool to confirm our finding, but early heart defects resulting in developmental compensation among the 20 connexins were a limitation (Reaume et al., 1995). On the other hand, mice carrying conditional knockout of Cx43 exhibited reduced size of the hippocampus, cortex, and cerebellum in postnatal animals, confirming our findings (Wiencken-Barger et al., 2007).

Thus, our *in vitro* and *in vivo* experiments indicate that spontaneous Ca^{2+} activity through gap junctions is involved in cellular processes that control the proliferation of neural progenitor cells. Blocking small-world networks with 1-octanol in differentiating neurons and in embryos reduced cortical thickness in the embryonic brain (Figure 10).

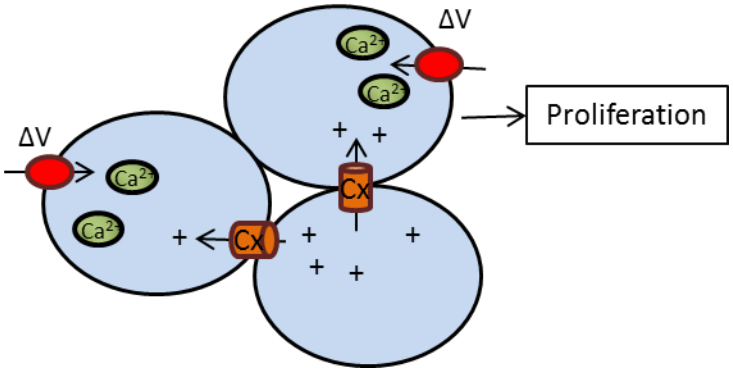

Figure 10: Schematic draft over Ca^{2+} signaling network through VOCs and gap junctions in neural progenitor cells that affects cell proliferation.

3.2 PAPER II: T α1h-CHANNEL-DEPENDENT SPONTANEOUS Ca^{2+}-ACTIVITY REGULATES NEURONAL DIFFERENTIATION THROUGH CASPASE-3

3.2.1 Differentiating neural stem cells exhibit spontaneous Ca^{2+} activity when they start to respond to depolarization

Mouse embryonic stem (mES) cells and fetal human neuroepithelial stem (hNS) cells were cultured in a defined medium (Shi et al., 2012; Ying et al., 2003) that allows fast and efficient monolayer production of neural progenitors and neurons. The neuroectodermal marker PAX6 and the progenitor marker nestin were both present from day 4 of differentiation of mES, at which the pluripotent marker Oct4 disappeared. Expression of βIII tubulin, a marker of immature neurons, increased from day 6. Ca^{2+} imaging was performed at days 0, 2, 4, 6, 8, and 10, and showed sparse spontaneous Ca^{2+} activity early in differentiation, while spontaneous Ca^{2+} activity significantly increased after 8 days. To test the ability of the cells to respond to depolarization during differentiation, cells were challenged with 50 mM KCl. Cells showed a prominent Ca^{2+} increase from day 6 onward.

3.2.2 A higher percentage of cells with spontaneous Ca^{2+} activity are positive for caspase-3 than non-active cells

To further investigate the role of spontaneous Ca^{2+} activity during neural differentiation, we performed back-tracing of single cells. Active cells were first detected with time-lapse Ca^{2+} imaging and thereafter fixed and post-immunostained for caspase-3. Both cells with high and low spontaneous activity were detected and the corresponding activated caspase-3 levels were analyzed. These experiments revealed that a higher number of cells with high levels of spontaneous activity were positive for caspase-3 compared to cells with low levels of calcium activity. Calcium activity has been associated with many physiological roles, as explained in the previous sections, but not directly with cell death. On the contrary, spontaneous activity mediated by synapses in newborn neurons has been associated with cell survival (Heck et al., 2008; Wagner-Golbs and Luhmann, 2012). However, recent findings reveal a new role for caspase-3 in differentiating neural progenitors from ES cells (Abdul-Ghani and Megeney, 2008; Fernando et al., 2005).

3.2.3 Expression of voltage-dependent Ca^{2+} channels varies during neuronal differentiation

Because the spontaneous Ca^{2+} activity coincides with the ability of neural progenitors to respond to depolarization treatments, we next investigated the expression pattern of all voltage-dependent PM Ca^{2+} channels with real-time PCR. At days 0, 2, 4, 6, 8, and 10, differentiating stem cells were examined for mRNA expression of Tg, Ld, Lc, R, N, Th, and P/Q Ca^{2+} channels. At day 8, the expression of T α1h VOCs increased dramatically and continued to increase by day 10.

3.2.4 Spontaneous Ca^{2+} activity is initiated by LVA

To study the impact of VOCs on spontaneous Ca^{2+} activity, cells at day 8 were challenged with various pharmacological inhibitors. Nickel, at a concentration specific for T α1h (30 µM) (Lee et al., 1999), almost completely blocked spontaneous activity, but failed to inhibit the response to depolarization. Mibefradil, a Ca^{2+} antagonist acting mainly on T-type Ca^{2+} channels, was used at two different concentrations: 3 µM and 30 µM. Both concentrations inhibited spontaneous activity but failed in inhibiting the response to KCl. Upon 3 µM mibefradil treatment, the KCl-induced Ca^{2+} transient was partially blocked, while upon 30 µM mibefradil, a concentration that inhibits both L and T Ca^{2+} channels (Ertel and Clozel, 1997), the Ca^{2+} response to depolarization was completely abolished.

3.2.5 Altering the open probability of T α1h VOCs affects enzymatic caspase-3 activity and mitochondrial membrane polarization

Next, the functional implications of T α1h VOCs were tested using hNS cells, since they are a more homogeneous system. hNS cells at day 4 of differentiation were treated for 6 h with 3 µM mibefradil or a low concentration of KCl (12 mM). These concentrations were chosen because they affect T α1h VOCs but not HVA Ca^{2+} channels. The caspase-3 and -7 inhibitor z-DEVD-FMK and the caspase-3 activator staurosporin were used as negative and positive controls, respectively. Mibefradil and z-DEVD-FMK treatments decreased significantly the enzymatic activity of caspase-3, while treatment with 12 mM KCl had no effect. As expected, staurosporin significantly increased enzymatic caspase-3 activity. TMRE, a positively charged dye that accumulates in active mitochondria with negatively charged membranes, was used to study the influence of mitochondria. The number of cells that

incorporated TMRE significantly increased when cells were pre-treated with mibefradil and z-DEVD-FMK, while cells pre-treated with 12 mM KCl showed a slight decrease in TMRE incorporation and staurosporin treatment significantly reduced the number of cells stained by TMRE. When apoptosis and necrosis were assessed by annexin-V and PI staining, only staurosporin significantly increased the number of apoptotic cells. These data indicate that altering T α1h VOC permeability affects enzymatic caspase-3 activity and mitochondrial membrane polarization without triggering apoptosis.

3.2.6 T α1h VOCs critically regulate caspase-3 and differentiation

The specific role of T α1h VOCs in neural differentiation was then investigated by performing lentiviral–mediated knock-down of T α1h VOC expression. Cells were transduced at day 1 of differentiation, when the spontaneous Ca^{2+} activity was present in these cells. Knock-down of T α1h VOC expression with lentiviral vectors resulted in a significant decrease in the expression of βIII tubulin RNA with a consistent decrease in the expression of T α1h VOCs. Caspase-3 enzymatic activity was also decreased in T α1h VOC knock-down cells. To examine whether activated caspase-3 was regulating cell death in our cell model, we next performed annexin-V staining. These experiments revealed no changes in cell death indicated by annexin-V. Caspase-3 levels were then assessed by immunostaining, and revealed decreased activation of capsase-3 in T α1h VOC knock-down cells. Over-expression of T α1h VOC led to a significant decrease in PAX6 expression and increase in βIII tubulin expression and caspase-3 enzymatic activity. The degree of over-expression was very strong at the mRNA level, with no differences in annexin-V staining. Levels of activated caspase-3 detected by immunostaining were also increased in T α1h VOC over-expressing cells.
Taken together, these results indicate that T α1h VOCs strongly affect caspase-3 activation and NS cell differentiation. Increases in cytosolic Ca^{2+} can activate both caspases and calpains, which regulate the processes of differentiation, apoptosis and necrosis. Fine regulation of caspases versus calpains activation may be the determining factor that decides cell fate (Chan and Mattson, 1999).

3.2.7 T α1h VOCs critically regulates embryonic brain development

To study the impact of VOC on NS cells in an *in vivo* setting, we examined brain development in mice. First, the VOC expression profile in the mouse forebrain was analyzed at different stages of development. At E9.5 the expression levels of VOC L and T were similar to that in differentiated

neural stem cells at day 4. The expression profiles of VOC L and T also showed correlation at between E14.5 and day 8 and between E15.5 and day 10.

Next, a T α1h VOC knockout mouse was used to examine the effect on brain architecture. Coronal sections of E14.5 brains were cut and immunostained for markers of neural differentiation. The expression levels of Tuj1 and MAP2 in the dorsal cortex were calculated in normal and knockout animals. Knockout mice showed a decrease in the thickness of the Tuj1 and MAP2 layer in the dorsal cortex. Furthermore, T α1h VOC knockout mice exhibited ventricular disruptions similar to those found in caspase-3, 9, Apaf1, and cytochrome c knockout animals (D'Amelio et al., 2010), suggesting that these molecules function in the same signaling pathway. Further analysis of T α1h VOC knockout animals revealed significant non-lethal cell abnormalities, including abnormal blood vessel morphology, cardiac fibrosis, and deficiencies in context-associated memory, as well as reduced size (Chen et al., 2003; Chen et al., 2012).

In summary, we report a novel signaling mechanism that connects Ca^{2+} entry through T α1h VOCs with caspase-3 activation and directs neural progenitor differentiation (Figure 11).

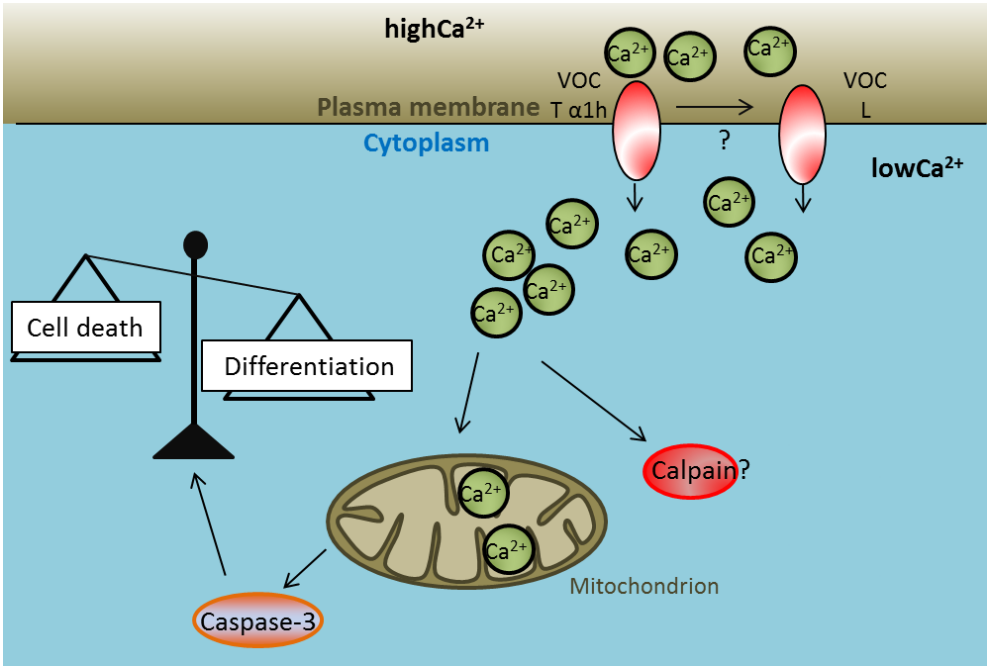

Figure 11: Schematic draft over Ca^{2+} signaling and caspase-3 activation that affects neural differentiation of neuroephitelial stem cells.

3.3 PAPER III: NON–DIOXIN-LIKE POLYCHLORINATED BIPHENYLS INTERFERE WITH NEURONAL DIFFERENTIATION OF EMBRYONIC NEURAL STEM CELLS

3.3.1 Non-cytotoxic concentrations of PCBs 153 and 180 enhance differentiation on neural stem cells

Neural stem cell cultures were used as a model to identify potential developmental neurotoxicants. Sub-lethal concentrations of PCB 153 and PCB 180 influence spontaneous differentiation of rat embryonic neural stem cells (NSCs). Both PCB 153 and PCB 180 induce a significant increase in the number of neurite-bearing Tuj1-positive cells with a concomitant decrease in cells expressing nestin, but no changes in the frequency of GFAP-positive cells or in necrotic or apoptotic cell death.

3.3.2 Exposure to PCBs 153 and 180 results in decreased neural stem cells proliferation

Cell cycle analysis was performed using FUCCI plasmids (Sakaue-Sawano et al., 2008) that encode fluorescent proteins, a system that allows different cell cycle phases to be distinguished. Depending on the protein level of Cdt1 and Geminin, which oscillate inversely (Cdt1 is accumulated in G1 phase and Geminin in S/G2/M phase), the cells express either a green or red fluorescence protein. The percentage of cells in S/G2/M phase (i.e., proliferating) decreased when treated with PCB 153 and 180. Further analysis with EdU staining confirmed this data.

3.3.3 PCBs decrease the number of cells with spontaneous Ca^{2+} activity

Micromolar concentrations of PCBs have been shown to disrupt Ca^{2+} homeostasis in many different cell types at different levels: alteration of basal Ca^{2+} concentrations, alteration of PKC activation, CAMKII functionality, or $InsP_3$ hydrolysis (Brown et al., 1998; Kodavanti et al., 1994). Moreover, it has been demonstrated that PCBs disrupt ryanodine sensitive release channels (Wong et al., 1997) and VOC-related Ca^{2+} signaling (Inglefield and Shafer, 2000). After 7 days of differentiation, cells were loaded with Fluo3-AM and Ca^{2+} imaging was performed. Exposure to PCB 153 or 180 decreased the number of cells showing spontaneous Ca^{2+} activity, in line with our observation of decreased differentiation. Spontaneous Ca^{2+} signals in undifferentiated cells can persist for many days, but they

become less frequent as the stem cells differentiate into fully differentiated and mature neurons. This has been shown in primary culture of mouse NS cells from embryonic day 14, in which both global and local spontaneous Ca^{2+} signals were shown to be more frequent during the early stages of neural precursor differentiation (Ciccolini et al., 2003). Moreover, both PCBs increased the number of glutamate responsive cells, demonstrating that the cells were more competent with regard to Ca^{2+} signaling.

3.3.4 Notch signaling is repressed by exposure to PCBs

The Notch signaling pathway has been implicated in a wide variety of essential cellular events, such as proliferation, migration, differentiation, and neurite outgrowth (Artavanis-Tsakonas et al., 1999). The expression of total Notch1 was analyzed by immunoblotting, revealing higher levels of expression in cells treated with PCBs 153 and 180. mRNA expression of the Notch target genes Hes5 (anti-neuronal) and Math1 (pro-neuronal) was also analyzed, revealing an upregulation of Hes5 and a downregulation of Math1 in PCB-treated cells.

In conclusion, non-cytotoxic nanomolar concentrations of both PCBs 153 and 180 interfere with the spontaneous neuronal differentiation of NSCs. NSCs appear to be a relevant model for *in vitro* neurotoxicity studies, and analysis of physiological events, such as cell proliferation and differentiation, are sensitive parameters by which to identify substances with potential developmental neurotoxicity.

3.4 PAPER IV: THE RET PLCγ PHOSPHOTYROSINE BINDING DOMAIN REGULATES Ca^{2+} SIGNALING AND NEOCORTICAL NEURONAL MIGRATION

3.4.1 Ca^{2+} signaling is affected by RET receptor activity

Hela cells were transfected with green fluorescent protein(GFP)-tagged wild-type RET and other RET constructs bearing point mutations of tyrosine residues at positions 1062, 1015, or both, to assess the effect of RET on Ca^{2+} signaling. Tyrosine 1015 was involved in the response to GDNF, which was oscillatory in 58% of the cases. The RET receptor-induced Ca^{2+} response to GDNF was dependent on PLCγ and internal Ca^{2+} stores. Tyr1015 is known to bind PLCγ to the RET receptor, suggesting that PLC plays a role in this signaling pathway. The response to GDNF was blocked by treatment with the PLC-inhibitor U73122 and in PLCγ knock-down cells. Further analysis showed that the Ca^{2+} response to GDNF was also blocked by 2-APB, an inhibitor of $InsP_3R$, but not by treatment with ryanodine or dandrolene, which are RyR inhibitors. Thapsigargin treatment, which depletes internal Ca^{2+} stores, completely inhibited the response to GDNF.

3.4.2 GDNF/RET-induced Ca^{2+} signaling phosphorylates ERK1/2 and CaMKII through Tyr 1015

Cells transfected with the wild-type RET and exposed to GDNF for 2 to 30 min showed a time-dependent increase in ERK1/2 phosphorylation, which was decreased in the presence of BAPTA, a cytosolic Ca^{2+} buffer. Mutation of Tyr1015 in RET severely reduced or abolished its ability to induce ERK1/2 and CAMKII phosphorylation. Thus, we demonstrated that the increase in Ca^{2+} after RET stimulation is important for ERK1/2 and CAMKII phosphorylation and that Tyr1015 plays a fundamental role in this signaling mechanism.

3.4.3 RET is expressed in the embryonic neocortex

The expression of RET in the embryonic neocortex was confirmed by immunohistochemistry, western blot, and real-time PCR analyses. Immunohistochemical analysis of mouse E14.5 brain coronal slices revealed homogenous RET expression in the VZ, the intermediated zone, and the cortical plate.

Quantification of RET expression was low compared to the positive control (cerebellum) but sufficient to have a physiological effect.

3.4.4 GDNF-stimulated neocortical progenitor migration in the developing brain is modulated by Tyr1015 in the RET receptor

Ex utero electroporation was performed to determine whether Tyr1015 plays a role in neocortical neuronal migration. Wild-type or the mutant RET constructs and were injected into the lateral ventricles of E14.5 embryonic forebrains, followed by culture of cortical slices. Beads soaked in GDNF were used to induce neuronal migration towards the cortical plate. The migration was inhibited by U73122 or by mutation of Tyr1015 in RET, demonstrating a role for Tyr1015 in neuronal migration induced by GDNF, as well as PLCγ. It was previously reported that GDNF/RET modulate differentiation and migration through different pathways, such as Ras/ERK in the case of enteric nervous system progenitors and PI3K/Akt in cortical GABAergic neurons (Natarajan et al., 2002; Pozas and Ibanez, 2005). This is the first report of direct stimulation of cytosolic Ca^{2+} release from the ER by RET signaling, through the PLCγ phosphotyrosine binding site Tyr1015 of RET. This Ca^{2+} increase then induces phosphorylation of the downstream effectors ERK1/2 and CaMKII (Figure 12).

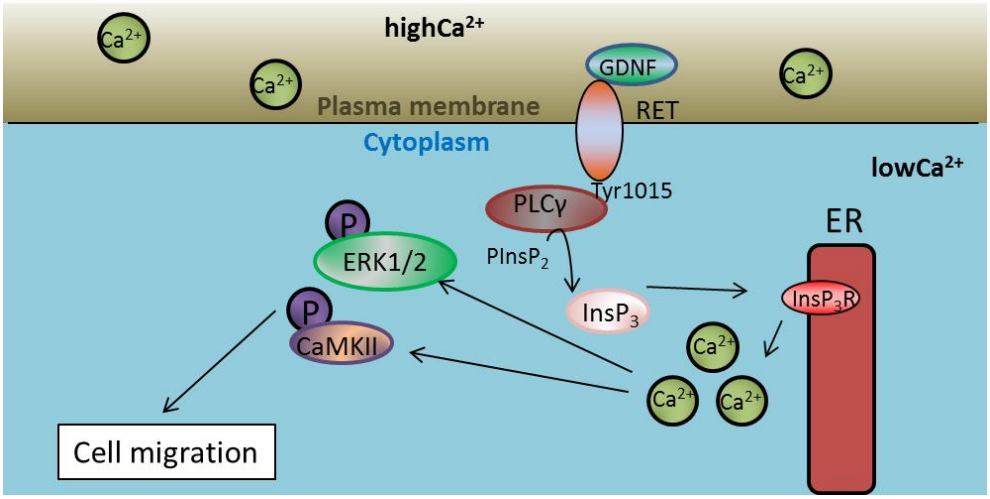

Figure 12: Schematic draft over Ca^{2+} signaling dependent on RET and PLCγ that interact through Tyr1015 and activate $InsP_3R$. Ca^{2+} released from ER leads to phosphorylation of ERK1/2 and CAMKII with consequences on neuronal migration.

4 GENERAL CONCLUSIONS

This thesis presents four studies on Ca^{2+} signaling that elucidate the major aspects of neurogenesis.

In paper I, using a multidisciplinary approach we demonstrate that immature cells in the developing brain organize in to small-world networks that critically regulate neural progenitor proliferation. Neural progenitors exhibit Ca^{2+} activity that is dependent on PM channels and gap junctions.

In paper II, we show that T α1h VOCs are highly expressed during neuronal development and promote spontaneous Ca^{2+} activity. Furthermore, T α1h VOCs strongly affect caspase-3 activation and NS cell differentiation, but not apoptosis.

In paper III, we proved that nanomolar concentrations of selected polychlorinated biphenyls interfere with neuronal spontaneous differentiation of NSCs through Notch signaling. Moreover, NSCs are validated as a valuable *in vitro* model for the identification of potential developmental neurotoxins.

In paper IV, we identified a novel RET mediated signaling pathway through RET Tyr1015 and PLCγ that leads to the elevation of cytosolic Ca^{2+} and phosphorylation of ERK1/2 and CaMKII and affects neuronal migration in the developing neocortex.

In conclusion, Ca^{2+} is an ubiquitous and versatile signaling messenger that regulates three of the most highly coordinated events in neurogenesis: proliferation, differentiation, and migration.

5 ACKNOWLEDGEMENTS

I would like to thank my supervisor **Per Uhlén** for accepting me in the lab and for always being very supportive of all my choices and my "bipolar" moments. It has been a long journey together and we have learnt a lot about different mentalities and attitudes! The freedom you granted me in your lab was precious; it gave me the possibility to fail a lot but also to learn so much and for sure to become a stronger person.

I thank my co-supervisors **Ernest Arenas** for always being available to help me with scientific advice and promising projects, **Seth Malmersjö** for teaching me the basis of Ca^{2+} imaging and **Carlos Villaescusa** for giving valuable scientific and moral support in the last part of my PhD.

I would like to thank Professor **Michael J. Berridge** for being my opponent. It is such a great honor!

I thank also Professor **Gilberto Fisone**, Professor **Erik Gylfe** and Professor **Jonas Muhr** for accepting to be my examination board despite all Lucia's related tasks.

Thanks to **Patrik Ernfors, Sten Linnarsson**, **Goncalo Castelo-Branco**, **Jens Hjerling-Leffler**, **Ulrika Marklund, Igor Adameyko** and **Tibor Harkany** for making Mol Neuro such a vibrant environment!

Uhlén's group, present and past

Thanks to **Shigeaki** for your great and humble help in all the embryo work, for organizing all our stuff and for your patience in teaching lab techniques; **Simone** for your sincere support and constructive critiques, **Cristian** for fruitful collaboration and for pushing me to give my best, **Erik** for great work with neural networks, **Ivar** for enduring conversations *på svenska*, **Teresa** for your true and contagious enthusiasm for science and tips with slice staining, **Songbai** for help with molecular biology and orders and for your enjoyable presentations, **Manuel** for bringing good cheer in the lab, **Staffan** for valuable medical recommendations and fear-of-flying support, **Göran** for the help at the microscope and the patience in handling my mass of data in the imaging pc, without giving in to the tempting solution to just cancel everything, **Nicolas** for your help in getting in to the lab, for teaching me so much and for the feedback on this thesis, **Marie** for your kindness and your helpful attitude especially in these last

months, **Hiromi** for teaching me cloning and for your hilarious way to be, **Antonio** for your positive support even from New Zealand.

.

Ernfors' group, present and past

Natalia for your sharp humor, for sharing being foodie and for giving me the occasion to re-discover Portugal, **Boris** for your true friendship even if covered with bitterness, your contagious hard working inclination and gossip sharing…and for the "nice" music you share with us in the lab, **Hind** for all the lunches and boxes supply and honest friendship, even if sometimes explosive, **Daohua** for insight into the Chinese medicine and culture, **Alessandro** for sharing the shame and discouragement regarding Italian politics, **Blanchi** for making every single moment spent with you in the lab so funny and for keeping order in our division, **Lili Li** for being a nice company in the ex-office, **Moritz** for being always prompt to organize events, **Dimitry** for help in the lab and for your fabulous photos, **Marina** for suggestions regarding the knockout mice**, Ruani** for your generosity and for helping me to get in the lab, **Sergi** for sharing FACS knowledge and disappointments and for so many useful suggestions, **Helena** for your firm belief and courage in maintaining safety rules and order in the lab (maybe it was not so evident but we really have appreciated that), **Isa** for nice time spent together and for your persistence, **Olga** for having brought new pets at Mol Neuro and for funny moments in the lab and in Portugal.

Arenas's group, present and past

I thank **Pia** for sincere friendship and mutual gym support, **Alca** for making me feeling so well that evening at haga parken and never stopping in inviting me to do activities (I really appreciate that!), **Carmen** for your smiling and kind South European inclination and for being a great boss of CCPD, **Spyros** for your stoicism…I am so jealous that nothing can bother you! Thanks **Lottie** for efficient organization of the chemicals, **Enrique** for the IWP compound, your humor and the 4-cokes expensive microwave, **Shanzeng** for useful suggestions regarding in utero electroporation and pipette preparations, **Geeta** for being a nice new office-mate, **Isabel MC** for patiently listening to my projects and for the feedback you gave me, **Catarina** for sharing so many lunches, brunches, dinners, PhD and life experiences and for being always so pro-active in organizing stuff, **Diogo** for the arguments in and outside the lab including our nice trip to Toronto, my mentor **Paola Sacchetti** for all the scientific and life suggestions, far but close to me! Thanks **Sandro** for the help with mES, for being a sincere friend away and for the great tour of Coimbra, **Emma** for helping me with E9.5 embryos' dissection (I

enjoyed the lesson a lot!), **Linda A** for your cheerfulness and generosity (I really hope to see you back one day), **Linda E** for funny lunch discussions, **Fabia** for your support in the lab and for leaving your great seat in the office to me. Good luck to you and Mark in Danmark!

Linnarsson's group, present and past

Hanna Jo for your strong determination in making things work and for keeping our minds always fresh in the office, **Hanna Ju** for being a calm and respectful office-mate, **Casper** for the mouse pad (it was a funny moment!), **Una** for the books and all useful suggestions about future careers, **Saiful** for sharing the pain of the last part of the PhD and for our nice trip to Toronto, **Pawel** for your friendship, many coffees and chat together in the old times and for introducing me to the Bandy world.

Harkany's group, present and past

Giuseppe for your humble and always positive inclination and help with the cortical measurements, **Daniela** for helping me to stand Boris in the office, **Roman** for patience in explaining electrophysiology to me, **Orsi** for the efficient maintenance of the cryostat. Good luck in Wien!

Adameyko's group

Nina for funny discussions in the office and for making me aware about privacy issues in the acknowledgements chapter, **Maryam** for nice lunch conversations and help with Swedish.

Castelo-Branco's group

Sueli for your sweetness, **Ana MF** for your great positive energy!

Hjerling-Leffler's group

Ana BM for your support in the lab and nice moments in Stockholm, **Hermany** for fruitful discussion about T channels and interesting stories from Brazil.

Johnny and **Alessandra**: Special mention for Mol Neuro's heart and mom, this division could not survive without you two! Thanks Johnny for your humble hard work and for the funny conversations in Swedish, always with a big smile on your face. Thanks Alessandra for your support and help for any kind of problem I had in all these years: I feel safe with you around!

Ex Mol Neuro

Thanks **Francois** for your hard and high quality work (it is nice to see that it can still lead to something in the modern fast-science), **Saida** for spending so much time in consoling me and trying to make me understand all the paths to go through towards a successful scientific career. Your determination and charm will make you such a great leader**!** Thanks **Mia** for trying to help me to find the committee members and for the funny chats in the cell culture room, **Anna O** for your acute contagious laughs, **Shermaine** and **Petra** for many funny moments in and outside the lab.

Collaborators

Thanks to **Michael Andäng** for introducing me to FACS**, Shaimaa, Kalle, Katsutoshi, Isabel L, Hampus, Evantia, Abdel El Manira and Gilad Silberberg** for fruitful collaborations**, Roshan, Wan** and **Sandra Ceccatelli** for giving me the opportunity to learn more about neurodevelopmental toxicology**, Michalina** for funny chats in front of the Ca^{2+} system, your fantastic biscuits and for teaching me so much about cortical neurons' signaling**, Ola Hermanson** for helping me in scientific disputes**, Anna Falk** for the hNS cells (I am so glad that you derived them!).

Södersjukhuset

Thanks to **Shahidul Islam** for giving me the opportunity to come to Sweden, for introducing me to the world of Ca^{2+} recording and for good advice at my half time! Thanks **Amanda, Kristina** and **Anna B** for being helpful and patient co-workers during my first period in Sweden.

Medical Product Agency

I would like to thank **Torbjörn Arvidsson** for letting me to work at MPA, **Ahmad Amini** for teaching me so much about mass spect in so little time and to **Annette Perolari** for taking care of me in the new lab.

Friends in Stockholm

Thanks **Nina** and **Santi** for all the fun that we had together **(**Santi, as you know I don't hate you out of the ski-slopes), **Angela** for all your advice regarding life, science and in organizing the PhD party, **Nicoletta, Antonio, Marta Mauro, Elena** and **Stefano** for being my Italy in Stockholm, for all the nice lunches, dinner, parties, support, and for reducing my homesickness a lot! **Ida** for great support

and friendship during the most sensitive part of my PhD, **Raffaele**, **Claudia** and **Francesca** for the fun at Fogdevreten and Jägargatan, **Paolo B** and **Korinna** for many nice moments included the bike trip in Utö, **Alessio DG** for all the help in Padua and in Stockholm (it was so comforting to have you here on the night of my arrival!), **Ana Cristina**, **Paolo S** and **Luca** for helping me live abroad in many ways.

Theater, present and past

Laura grazie per l'appoggio artistico, i "sermoni", le risate e le chiacchierate davanti a un caffè, thanks **Silvia** for Herman and for your funny uncontrolled laugh, **Christian** for your friendship in the crazy Jägargatan's old times… we still miss you a lot! Thanks **Elisa M** for your sincere support during all these years, for sharing many sorrows but also many funny moments and for coming to Bologna… it was so nice to see you there! Thanks **Simone** and **Marianna** for all the movies and the great moments spent in Stockholm and in Rome, **Giulio** for sharing your great expertise in apoptosis and caspases and for showing us the "best" pubs of Stockholm, **Elisa S** for your everlasting enthusiasm, **Giuseppe G, Chiara T, Chiara B, Flaminia** and **Alessandro** for many funny moments on the stage, **Maddalena** for joining the group!

Family and friends in Italy

Grazie **mamma** per avermi sempre supportato (e sopportato) in tutto quello che faccio. Certo non avevi scelta, ma sei stata brava a non farlo vedere! **Papà,** sei il motivo per cui sono qui a fare quello che sto facendo, peccato tu non sia voluto venire; grazie **Mirco** per tutte le visite, i consigli e il supporto morale in tutti questi anni a distanza! **Barbara,** la più grande fan di Stoccolma, grazie per aver trascinato qui Mirco e mamma così tante volte! Grazie **Ilenia** per la duratura amicizia, ma basta postare foto dalle Hawaii (è pura crudeltà verso chi vive in Svezia!), **Fede, Mery e Irene** per le tante serate passate insieme e l'efficiente organizzazione di eventi ogni volta che ritorno in Italia, **Elena** per tutte le nostre discussioni riguardo futuro, presente, passato, poesia, affari, letteratura e universo, **Sara, Maddi, Elisa S** e **Stefano** per le avventure e gli spritz del mercoledì patavino, **Chiara, Matteo, Alberto, Alessio T, Elisa B e Alice** grazie per tutti i bei momenti passati al T.L.Caro e per essere ancora così vicini dopo tanti anni.Vi adoro!

Family in Stockholm

Last but not at all least: for all the patience, all the support, all the fun during this tough period… thanks **Alba**! And of course thanks **Roberto** for being always here close to me. I am joking, even if

Alba was a great company during my thesis writing, this PhD is, in part, also yours Robi! The patience and the endurance that you demonstrated during these 5 years are the proof that you are going to be a fantastic and already well trained psychologist, and that I am a very lucky person! Thanks thanks thanks!

6 REFERENCES

Abdul-Ghani, M., and Megeney, L.A. (2008). Rehabilitation of a contract killer: caspase-3 directs stem cell differentiation. Cell Stem Cell *2*, 515-516.

Abranches, E., Silva, M., Pradier, L., Schulz, H., Hummel, O., Henrique, D., and Bekman, E. (2009). Neural differentiation of embryonic stem cells in vitro: a road map to neurogenesis in the embryo. PLoS One *4*, e6286.

Adinolfi, M. (1985). The development of the human blood-CSF-brain barrier. Dev Med Child Neurol *27*, 532-537.

Aizawa, H., Hu, S.C., Bobb, K., Balakrishnan, K., Ince, G., Gurevich, I., Cowan, M., and Ghosh, A. (2004). Dendrite development regulated by CREST, a calcium-regulated transcriptional activator. Science *303*, 197-202.

Aizman, O., Uhlen, P., Lal, M., Brismar, H., and Aperia, A. (2001). Ouabain, a steroid hormone that signals with slow calcium oscillations. Proc Natl Acad Sci U S A *98*, 13420-13424.

Ariano, P., Dalmazzo, S., Owsianik, G., Nilius, B., and Lovisolo, D. (2011). TRPC channels are involved in calcium-dependent migration and proliferation in immortalized GnRH neurons. Cell Calcium *49*, 387-394.

Artavanis-Tsakonas, S., Rand, M.D., and Lake, R.J. (1999). Notch signaling: cell fate control and signal integration in development. Science *284*, 770-776.

Bading, H., Ginty, D.D., and Greenberg, M.E. (1993). Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. Science *260*, 181-186.

Bain, G., Kitchens, D., Yao, M., Huettner, J.E., and Gottlieb, D.I. (1995). Embryonic stem cells express neuronal properties in vitro. Dev Biol *168*, 342-357.

Bean, B.P., and McDonough, S.I. (1998). Two for T. Neuron *20*, 825-828.

Bennett, M.V., and Zukin, R.S. (2004). Electrical coupling and neuronal synchronization in the Mammalian brain. Neuron *41*, 495-511.

Berridge, M.J., Bootman, M.D., and Lipp, P. (1998). Calcium--a life and death signal. Nature *395*, 645-648.

Bezprozvanny, I., Watras, J., and Ehrlich, B.E. (1991). Bell-shaped calcium-response curves of Ins(1,4,5)P3- and calcium-gated channels from endoplasmic reticulum of cerebellum. Nature *351*, 751-754.

Bootman, M., Niggli, E., Berridge, M., and Lipp, P. (1997). Imaging the hierarchical Ca2+ signalling system in HeLa cells. J Physiol *499 (Pt 2)*, 307-314.

Bootman, M.D., and Berridge, M.J. (1995). The elemental principles of calcium signaling. Cell *83*, 675-678.

Borodinsky, L.N., O'Leary, D., Neale, J.H., Vicini, S., Coso, O.A., and Fiszman, M.L. (2003). GABA-induced neurite outgrowth of cerebellar granule cells is mediated by GABA(A) receptor activation, calcium influx and CaMKII and erk1/2 pathways. J Neurochem *84*, 1411-1420.

Borodinsky, L.N., Root, C.M., Cronin, J.A., Sann, S.B., Gu, X., and Spitzer, N.C. (2004). Activity-dependent homeostatic specification of transmitter expression in embryonic neurons. Nature *429*, 523-530.

Brown, A.P., Olivero-Verbel, J., Holdan, W.L., and Ganey, P.E. (1998). Neutrophil activation by polychlorinated biphenyls: structure-activity relationship. Toxicol Sci *46*, 308-316.

Capiod, T. (2011). Cell proliferation, calcium influx and calcium channels. Biochimie *93*, 2075-2079.

Capiod, T. (2013). The need for calcium channels in cell proliferation. Recent Pat Anticancer Drug Discov *8*, 4-17.

Carafoli, E., Santella, L., Branca, D., and Brini, M. (2001). Generation, control, and processing of cellular calcium signals. Crit Rev Biochem Mol Biol *36*, 107-260.
Carrion, A.M., Link, W.A., Ledo, F., Mellstrom, B., and Naranjo, J.R. (1999). DREAM is a Ca2+-regulated transcriptional repressor. Nature *398*, 80-84.
Catterall, W.A. (2011). Voltage-gated calcium channels. Cold Spring Harb Perspect Biol *3*, a003947.
Chan, S.L., and Mattson, M.P. (1999). Caspase and calpain substrates: roles in synaptic plasticity and cell death. J Neurosci Res *58*, 167-190.
Chemin, J., Nargeot, J., and Lory, P. (2002). Neuronal T-type alpha 1H calcium channels induce neuritogenesis and expression of high-voltage-activated calcium channels in the NG108-15 cell line. J Neurosci *22*, 6856-6862.
Chen, C.C., Lamping, K.G., Nuno, D.W., Barresi, R., Prouty, S.J., Lavoie, J.L., Cribbs, L.L., England, S.K., Sigmund, C.D., Weiss, R.M., *et al.* (2003). Abnormal coronary function in mice deficient in alpha1H T-type Ca2+ channels. Science *302*, 1416-1418.
Chen, C.C., Shen, J.W., Chung, N.C., Min, M.Y., Cheng, S.J., and Liu, I.Y. (2012). Retrieval of context-associated memory is dependent on the Ca(v)3.2 T-type calcium channel. PLoS One *7*, e29384.
Choe, C.U., and Ehrlich, B.E. (2006). The inositol 1,4,5-trisphosphate receptor (IP3R) and its regulators: sometimes good and sometimes bad teamwork. Sci STKE *2006*, re15.
Chung, S.C., McDonald, T.V., and Gardner, P. (1994). Inhibition by SK&F 96365 of Ca2+ current, IL-2 production and activation in T lymphocytes. Br J Pharmacol *113*, 861-868.
Ciccolini, F., Collins, T.J., Sudhoelter, J., Lipp, P., Berridge, M.J., and Bootman, M.D. (2003). Local and global spontaneous calcium events regulate neurite outgrowth and onset of GABAergic phenotype during neural precursor differentiation. J Neurosci *23*, 103-111.
Cina, C., Bechberger, J.F., Ozog, M.A., and Naus, C.C. (2007). Expression of connexins in embryonic mouse neocortical development. J Comp Neurol *504*, 298-313.
Clipstone, N.A., and Crabtree, G.R. (1992). Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. Nature *357*, 695-697.
Crunelli, V., Toth, T.I., Cope, D.W., Blethyn, K., and Hughes, S.W. (2005). The 'window' T-type calcium current in brain dynamics of different behavioural states. J Physiol *562*, 121-129.
D'Amelio, M., Cavallucci, V., and Cecconi, F. (2010). Neuronal caspase-3 signaling: not only cell death. Cell Death Differ *17*, 1104-1114.
D'Amelio, M., Cavallucci, V., Middei, S., Marchetti, C., Pacioni, S., Ferri, A., Diamantini, A., De Zio, D., Carrara, P., Battistini, L., *et al.* (2011). Caspase-3 triggers early synaptic dysfunction in a mouse model of Alzheimer's disease. Nat Neurosci *14*, 69-76.
Darcy, D.P., and Isaacson, J.S. (2009). L-type calcium channels govern calcium signaling in migrating newborn neurons in the postnatal olfactory bulb. J Neurosci *29*, 2510-2518.
De Koninck, P., and Schulman, H. (1998). Sensitivity of CaM kinase II to the frequency of Ca2+ oscillations. Science *279*, 227-230.
Desfrere, L., Karlsson, M., Hiyoshi, H., Malmersjo, S., Nanou, E., Estrada, M., Miyakawa, A., Lagercrantz, H., El Manira, A., Lal, M., *et al.* (2009). Na,K-ATPase signal transduction triggers CREB activation and dendritic growth. Proc Natl Acad Sci U S A *106*, 2212-2217.
Dolmetsch, R.E., Lewis, R.S., Goodnow, C.C., and Healy, J.I. (1997). Differential activation of transcription factors induced by Ca2+ response amplitude and duration. Nature *386*, 855-858.
Dolmetsch, R.E., Xu, K., and Lewis, R.S. (1998). Calcium oscillations increase the efficiency and specificity of gene expression. Nature *392*, 933-936.
Elias, L.A., and Kriegstein, A.R. (2008). Gap junctions: multifaceted regulators of embryonic cortical development. Trends Neurosci *31*, 243-250.

Elias, L.A., Wang, D.D., and Kriegstein, A.R. (2007). Gap junction adhesion is necessary for radial migration in the neocortex. Nature *448*, 901-907.
Enfissi, A., Prigent, S., Colosetti, P., and Capiod, T. (2004). The blocking of capacitative calcium entry by 2-aminoethyl diphenylborate (2-APB) and carboxyamidotriazole (CAI) inhibits proliferation in Hep G2 and Huh-7 human hepatoma cells. Cell Calcium *36*, 459-467.
Ertel, E.A., Campbell, K.P., Harpold, M.M., Hofmann, F., Mori, Y., Perez-Reyes, E., Schwartz, A., Snutch, T.P., Tanabe, T., Birnbaumer, L., *et al.* (2000). Nomenclature of voltage-gated calcium channels. Neuron *25*, 533-535.
Ertel, S.I., and Clozel, J.P. (1997). Mibefradil (Ro 40-5967): the first selective T-type Ca2+ channel blocker. Expert Opin Investig Drugs *6*, 569-582.
Estrada, M., Uhlen, P., and Ehrlich, B.E. (2006). Ca2+ oscillations induced by testosterone enhance neurite outgrowth. J Cell Sci *119*, 733-743.
Evans, W.H., and Martin, P.E. (2002). Gap junctions: structure and function (Review). Mol Membr Biol *19*, 121-136.
Fan, W., Dai, Y., Xu, H., Zhu, X., Cai, P., Wang, L., Sun, C., Hu, C., Zheng, P., and Zhao, B.Q. (2013). Caspase-3 Modulates Regenerative Response after Stroke. Stem Cells.
Fernando, P., Brunette, S., and Megeney, L.A. (2005). Neural stem cell differentiation is dependent upon endogenous caspase 3 activity. FASEB J *19*, 1671-1673.
Feske, S. (2007). Calcium signalling in lymphocyte activation and disease. Nat Rev Immunol *7*, 690-702.
Fink, C.C., Bayer, K.U., Myers, J.W., Ferrell, J.E., Jr., Schulman, H., and Meyer, T. (2003). Selective regulation of neurite extension and synapse formation by the beta but not the alpha isoform of CaMKII. Neuron *39*, 283-297.
Fleckenstein, A., Janke, J., Doring, H.J., and Leder, O. (1974). Myocardial fiber necrosis due to intracellular Ca overload-a new principle in cardiac pathophysiology. Recent Adv Stud Cardiac Struct Metab *4*, 563-580.
Fransson, P., Aden, U., Blennow, M., and Lagercrantz, H. (2011). The functional architecture of the infant brain as revealed by resting-state fMRI. Cereb Cortex *21*, 145-154.
Fujita, J., Crane, A.M., Souza, M.K., Dejosez, M., Kyba, M., Flavell, R.A., Thomson, J.A., and Zwaka, T.P. (2008). Caspase activity mediates the differentiation of embryonic stem cells. Cell Stem Cell *2*, 595-601.
Futatsugi, A., Nakamura, T., Yamada, M.K., Ebisui, E., Nakamura, K., Uchida, K., Kitaguchi, T., Takahashi-Iwanaga, H., Noda, T., Aruga, J., *et al.* (2005). IP3 receptor types 2 and 3 mediate exocrine secretion underlying energy metabolism. Science *309*, 2232-2234.
Gaspard, N., Bouschet, T., Herpoel, A., Naeije, G., van den Ameele, J., and Vanderhaeghen, P. (2009). Generation of cortical neurons from mouse embryonic stem cells. Nat Protoc *4*, 1454-1463.
Guan, C.B., Xu, H.T., Jin, M., Yuan, X.B., and Poo, M.M. (2007). Long-range Ca2+ signaling from growth cone to soma mediates reversal of neuronal migration induced by slit-2. Cell *129*, 385-395.
Gudermann, T., Hofmann, T., Mederos y Schnitzler, M., and Dietrich, A. (2004). Activation, subunit composition and physiological relevance of DAG-sensitive TRPC proteins. Novartis Found Symp *258*, 103-118; discussion 118-122, 155-109, 263-106.
Guo, Z., Shi, F., Zhang, L., Zhang, H., Yang, J., Li, B., Jia, J., and Wang, X. (2010). Critical role of L-type voltage-dependent Ca2+ channels in neural progenitor cell proliferation induced by hypoxia. Neurosci Lett *478*, 156-160.
Guroff, G. (1964). A Neutral, Calcium-Activated Proteinase from the Soluble Fraction of Rat Brain. J Biol Chem *239*, 149-155.

Heck, N., Golbs, A., Riedemann, T., Sun, J.J., Lessmann, V., and Luhmann, H.J. (2008). Activity-dependent regulation of neuronal apoptosis in neonatal mouse cerebral cortex. Cereb Cortex *18*, 1335-1349.

Hitchcock, S.E. (1975). Regulation of muscle contraction: bindings of troponin and its components to actin and tropomyosin. Eur J Biochem *52*, 255-263.

Hodgkin, A.L., and Keynes, R.D. (1957). Movements of labelled calcium in squid giant axons. J Physiol *138*, 253-281.

Inglefield, J.R., and Shafer, T.J. (2000). Polychlorinated biphenyl-stimulation of Ca(2+) oscillations in developing neocortical cells: a role for excitatory transmitters and L-type voltage-sensitive Ca(2+) channels. J Pharmacol Exp Ther *295*, 105-113.

Iwai, M., Michikawa, T., Bosanac, I., Ikura, M., and Mikoshiba, K. (2007). Molecular basis of the isoform-specific ligand-binding affinity of inositol 1,4,5-trisphosphate receptors. J Biol Chem *282*, 12755-12764.

Kahl, C.R., and Means, A.R. (2003). Regulation of cell cycle progression by calcium/calmodulin-dependent pathways. Endocr Rev *24*, 719-736.

Kandler, K., and Katz, L.C. (1998). Coordination of neuronal activity in developing visual cortex by gap junction-mediated biochemical communication. J Neurosci *18*, 1419-1427.

Kapur, N., Mignery, G.A., and Banach, K. (2007). Cell cycle-dependent calcium oscillations in mouse embryonic stem cells. Am J Physiol Cell Physiol *292*, C1510-1518.

Kawasaki, H., Mizuseki, K., Nishikawa, S., Kaneko, S., Kuwana, Y., Nakanishi, S., Nishikawa, S.I., and Sasai, Y. (2000). Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity. Neuron *28*, 31-40.

Kennedy, M.B. (1993). The postsynaptic density. Curr Opin Neurobiol *3*, 732-737.

Kodavanti, P.R., Shafer, T.J., Ward, T.R., Mundy, W.R., Freudenrich, T., Harry, G.J., and Tilson, H.A. (1994). Differential effects of polychlorinated biphenyl congeners on phosphoinositide hydrolysis and protein kinase C translocation in rat cerebellar granule cells. Brain Res *662*, 75-82.

Komuro, H., and Kumada, T. (2005). Ca2+ transients control CNS neuronal migration. Cell Calcium *37*, 387-393.

Komuro, H., and Rakic, P. (1992). Selective role of N-type calcium channels in neuronal migration. Science *257*, 806-809.

Komuro, H., and Rakic, P. (1993). Modulation of neuronal migration by NMDA receptors. Science *260*, 95-97.

Komuro, H., and Rakic, P. (1996). Intracellular Ca2+ fluctuations modulate the rate of neuronal migration. Neuron *17*, 275-285.

Lacinova, L. (2005). Voltage-dependent calcium channels. Gen Physiol Biophys *24 Suppl 1*, 1-78.

Leclerc, C., Daguzan, C., Nicolas, M.T., Chabret, C., Duprat, A.M., and Moreau, M. (1997). L-type calcium channel activation controls the in vivo transduction of the neuralizing signal in the amphibian embryos. Mech Dev *64*, 105-110.

Leclerc, C., Neant, I., and Moreau, M. (2011). Early neural development in vertebrates is also a matter of calcium. Biochimie *93*, 2102-2111.

Leclerc, C., Neant, I., and Moreau, M. (2012). The calcium: an early signal that initiates the formation of the nervous system during embryogenesis. Front Mol Neurosci *5*, 3.

Lee, J.H., Gomora, J.C., Cribbs, L.L., and Perez-Reyes, E. (1999). Nickel block of three cloned T-type calcium channels: low concentrations selectively block alpha1H. Biophys J *77*, 3034-3042.

Li, L., Stefan, M.I., and Le Novere, N. (2012). Calcium input frequency, duration and amplitude differentially modulate the relative activation of calcineurin and CaMKII. PLoS One *7*, e43810.

Li, M., Pevny, L., Lovell-Badge, R., and Smith, A. (1998). Generation of purified neural precursors from embryonic stem cells by lineage selection. Curr Biol *8*, 971-974.
Lin, H.H., Bell, E., Uwanogho, D., Perfect, L.W., Noristani, H., Bates, T.J., Snetkov, V., Price, J., and Sun, Y.M. (2010). Neuronatin promotes neural lineage in ESCs via Ca(2+) signaling. Stem Cells *28*, 1950-1960.
Lohmann, C., Myhr, K.L., and Wong, R.O. (2002). Transmitter-evoked local calcium release stabilizes developing dendrites. Nature *418*, 177-181.
Louhivuori, L.M., Louhivuori, V., Wigren, H.K., Hakala, E., Jansson, L.C., Nordstrom, T., Castren, M.L., and Akerman, K.E. (2013). Role of low voltage activated calcium channels in neuritogenesis and active migration of embryonic neural progenitor cells. Stem Cells Dev *22*, 1206-1219.
Lovisolo, D., Ariano, P., and Distasi, C. (2012). Calcium signaling in neuronal motility: pharmacological tools for investigating specific pathways. Curr Med Chem *19*, 5793-5801.
Lynn, J.W., and Chambers, E.L. (1984). Voltage clamp studies of fertilization in sea urchin eggs. I. Effect of clamped membrane potential on sperm entry, activation, and development. Dev Biol *102*, 98-109.
Marambaud, P., Dreses-Werringloer, U., and Vingtdeux, V. (2009). Calcium signaling in neurodegeneration. Mol Neurodegener *4*, 20.
Matsumoto, M., Nakagawa, T., Inoue, T., Nagata, E., Tanaka, K., Takano, H., Minowa, O., Kuno, J., Sakakibara, S., Yamada, M., *et al.* (1996). Ataxia and epileptic seizures in mice lacking type 1 inositol 1,4,5-trisphosphate receptor. Nature *379*, 168-171.
Mendes, C.C., Gomes, D.A., Thompson, M., Souto, N.C., Goes, T.S., Goes, A.M., Rodrigues, M.A., Gomez, M.V., Nathanson, M.H., and Leite, M.F. (2005). The type III inositol 1,4,5-trisphosphate receptor preferentially transmits apoptotic Ca2+ signals into mitochondria. J Biol Chem *280*, 40892-40900.
Miao, Y.L., Stein, P., Jefferson, W.N., Padilla-Banks, E., and Williams, C.J. (2012). Calcium influx-mediated signaling is required for complete mouse egg activation. Proc Natl Acad Sci U S A *109*, 4169-4174.
Mikoshiba, K. (2007). IP3 receptor/Ca2+ channel: from discovery to new signaling concepts. J Neurochem *102*, 1426-1446.
Miyazaki, S., Shirakawa, H., Nakada, K., and Honda, Y. (1993). Essential role of the inositol 1,4,5-trisphosphate receptor/Ca2+ release channel in Ca2+ waves and Ca2+ oscillations at fertilization of mammalian eggs. Dev Biol *158*, 62-78.
Miyazaki, S., Yuzaki, M., Nakada, K., Shirakawa, H., Nakanishi, S., Nakade, S., and Mikoshiba, K. (1992). Block of Ca2+ wave and Ca2+ oscillation by antibody to the inositol 1,4,5-trisphosphate receptor in fertilized hamster eggs. Science *257*, 251-255.
Nadarajah, B., Jones, A.M., Evans, W.H., and Parnavelas, J.G. (1997). Differential expression of connexins during neocortical development and neuronal circuit formation. J Neurosci *17*, 3096-3111.
Natarajan, D., Marcos-Gutierrez, C., Pachnis, V., and de Graaff, E. (2002). Requirement of signalling by receptor tyrosine kinase RET for the directed migration of enteric nervous system progenitor cells during mammalian embryogenesis. Development *129*, 5151-5160.
Nusse, O., Serrander, L., Lew, D.P., and Krause, K.H. (1998). Ca2+-induced exocytosis in individual human neutrophils: high- and low-affinity granule populations and submaximal responses. EMBO J *17*, 1279-1288.
Orrenius, S., Zhivotovsky, B., and Nicotera, P. (2003). Regulation of cell death: the calcium-apoptosis link. Nat Rev Mol Cell Biol *4*, 552-565.

Panner, A., and Wurster, R.D. (2006). T-type calcium channels and tumor proliferation. Cell Calcium *40*, 253-259.
Platel, J.C., Dave, K.A., and Bordey, A. (2008). Control of neuroblast production and migration by converging GABA and glutamate signals in the postnatal forebrain. J Physiol *586*, 3739-3743.
Pozas, E., and Ibanez, C.F. (2005). GDNF and GFRalpha1 promote differentiation and tangential migration of cortical GABAergic neurons. Neuron *45*, 701-713.
Pregno, G., Zamburlin, P., Gambarotta, G., Farcito, S., Licheri, V., Fregnan, F., Perroteau, I., Lovisolo, D., and Bovolin, P. (2011). Neuregulin1/ErbB4-induced migration in ST14A striatal progenitors: calcium-dependent mechanisms and modulation by NMDA receptor activation. BMC Neurosci *12*, 103.
Qian, X., Shen, Q., Goderie, S.K., He, W., Capela, A., Davis, A.A., and Temple, S. (2000). Timing of CNS cell generation: a programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. Neuron *28*, 69-80.
Reaume, A.G., de Sousa, P.A., Kulkarni, S., Langille, B.L., Zhu, D., Davies, T.C., Juneja, S.C., Kidder, G.M., and Rossant, J. (1995). Cardiac malformation in neonatal mice lacking connexin43. Science *267*, 1831-1834.
Redmond, L., Kashani, A.H., and Ghosh, A. (2002). Calcium regulation of dendritic growth via CaM kinase IV and CREB-mediated transcription. Neuron *34*, 999-1010.
Roderick, H.L., and Cook, S.J. (2008). Ca2+ signalling checkpoints in cancer: remodelling Ca2+ for cancer cell proliferation and survival. Nat Rev Cancer *8*, 361-375.
Rohn, T.T., Cusack, S.M., Kessinger, S.R., and Oxford, J.T. (2004). Caspase activation independent of cell death is required for proper cell dispersal and correct morphology in PC12 cells. Exp Cell Res *295*, 215-225.
Salvesen, G.S., and Riedl, S.J. (2008). Caspase mechanisms. Adv Exp Med Biol *615*, 13-23.
Sandow, A. (1950). The latent period of muscular contraction. Arch Phys Med Rehabil *31*, 367-377.
Schumacher, J.A., Hsieh, Y.W., Chen, S., Pirri, J.K., Alkema, M.J., Li, W.H., Chang, C., and Chuang, C.F. (2012). Intercellular calcium signaling in a gap junction-coupled cell network establishes asymmetric neuronal fates in C. elegans. Development *139*, 4191-4201.
Schwaninger, M., Blume, R., Kruger, M., Lux, G., Oetjen, E., and Knepel, W. (1995). Involvement of the Ca(2+)-dependent phosphatase calcineurin in gene transcription that is stimulated by cAMP through cAMP response elements. J Biol Chem *270*, 8860-8866.
Shi, Y., Kirwan, P., Smith, J., Robinson, H.P., and Livesey, F.J. (2012). Human cerebral cortex development from pluripotent stem cells to functional excitatory synapses. Nat Neurosci *15*, 477-486, S471.
Snutch, T.P., Leonard, J.P., Gilbert, M.M., Lester, H.A., and Davidson, N. (1990). Rat brain expresses a heterogeneous family of calcium channels. Proc Natl Acad Sci U S A *87*, 3391-3395.
Sparrow, M.P., Mrwa, U., Hofmann, F., and Ruegg, J.C. (1981). Calmodulin is essential for smooth muscle contraction. FEBS Lett *125*, 141-145.
Spitzer, N.C. (2012). Activity-dependent neurotransmitter respecification. Nat Rev Neurosci *13*, 94-106.
Spitzer, N.C., Borodinsky, L.N., and Root, C.M. (2005). Homeostatic activity-dependent paradigm for neurotransmitter specification. Cell Calcium *37*, 417-423.
Stavridis, M.P., Lunn, J.S., Collins, B.J., and Storey, K.G. (2007). A discrete period of FGF-induced Erk1/2 signalling is required for vertebrate neural specification. Development *134*, 2889-2894.
Storch, U., Forst, A.L., Philipp, M., Gudermann, T., and Mederos y Schnitzler, M. (2012). Transient receptor potential channel 1 (TRPC1) reduces calcium permeability in heteromeric channel complexes. J Biol Chem *287*, 3530-3540.

Streb, H., Irvine, R.F., Berridge, M.J., and Schulz, I. (1983). Release of Ca2+ from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. Nature *306*, 67-69.
Sudhof, T.C., and Rizo, J. (1996). Synaptotagmins: C2-domain proteins that regulate membrane traffic. Neuron *17*, 379-388.
Taylor, J.M., and Simpson, R.U. (1992). Inhibition of cancer cell growth by calcium channel antagonists in the athymic mouse. Cancer Res *52*, 2413-2418.
Tilson, H.A., MacPhail, R.C., and Crofton, K.M. (1995). Defining neurotoxicity in a decision-making context. Neurotoxicology *16*, 363-375.
Toba, Y., Pakiam, J.G., and Wray, S. (2005). Voltage-gated calcium channels in developing GnRH-1 neuronal system in the mouse. Eur J Neurosci *22*, 79-92.
Tsien, R.W., Lipscombe, D., Madison, D.V., Bley, K.R., and Fox, A.P. (1988). Multiple types of neuronal calcium channels and their selective modulation. Trends Neurosci *11*, 431-438.
Tu, H., Wang, Z., and Bezprozvanny, I. (2005). Modulation of mammalian inositol 1,4,5-trisphosphate receptor isoforms by calcium: a role of calcium sensor region. Biophys J *88*, 1056-1069.
Uhlen, P., and Fritz, N. (2010). Biochemistry of calcium oscillations. Biochem Biophys Res Commun *396*, 28-32.
Uhlen, P., Laestadius, A., Jahnukainen, T., Soderblom, T., Backhed, F., Celsi, G., Brismar, H., Normark, S., Aperia, A., and Richter-Dahlfors, A. (2000). Alpha-haemolysin of uropathogenic E. coli induces Ca2+ oscillations in renal epithelial cells. Nature *405*, 694-697.
Vaillant, A.R., Zanassi, P., Walsh, G.S., Aumont, A., Alonso, A., and Miller, F.D. (2002). Signaling mechanisms underlying reversible, activity-dependent dendrite formation. Neuron *34*, 985-998.
Vanlangenakker, N., Vanden Berghe, T., Krysko, D.V., Festjens, N., and Vandenabeele, P. (2008). Molecular mechanisms and pathophysiology of necrotic cell death. Curr Mol Med *8*, 207-220.
Varnai, P., Hunyady, L., and Balla, T. (2009). STIM and Orai: the long-awaited constituents of store-operated calcium entry. Trends Pharmacol Sci *30*, 118-128.
Wagner-Golbs, A., and Luhmann, H.J. (2012). Activity-dependent survival of developing neocortical neurons depends on PI3K signalling. J Neurochem *120*, 495-501.
Wagner, L.E., 2nd, and Yule, D.I. (2012). Differential regulation of the InsP(3) receptor type-1 and -2 single channel properties by InsP(3), Ca(2)(+) and ATP. J Physiol *590*, 3245-3259.
Wang, S.Q., Wei, C., Zhao, G., Brochet, D.X., Shen, J., Song, L.S., Wang, W., Yang, D., and Cheng, H. (2004). Imaging microdomain Ca2+ in muscle cells. Circ Res *94*, 1011-1022.
Weissman, T.A., Riquelme, P.A., Ivic, L., Flint, A.C., and Kriegstein, A.R. (2004). Calcium waves propagate through radial glial cells and modulate proliferation in the developing neocortex. Neuron *43*, 647-661.
Wheeler, D.G., Barrett, C.F., Groth, R.D., Safa, P., and Tsien, R.W. (2008). CaMKII locally encodes L-type channel activity to signal to nuclear CREB in excitation-transcription coupling. J Cell Biol *183*, 849-863.
White, B.A. (1985). Evidence for a role of calmodulin in the regulation of prolactin gene expression. J Biol Chem *260*, 1213-1217.
Wiencken-Barger, A.E., Djukic, B., Casper, K.B., and McCarthy, K.D. (2007). A role for Connexin43 during neurodevelopment. Glia *55*, 675-686.
Wong, P.W., Brackney, W.R., and Pessah, I.N. (1997). Ortho-substituted polychlorinated biphenyls alter microsomal calcium transport by direct interaction with ryanodine receptors of mammalian brain. J Biol Chem *272*, 15145-15153.
Wu, G.Y., Deisseroth, K., and Tsien, R.W. (2001). Spaced stimuli stabilize MAPK pathway activation and its effects on dendritic morphology. Nat Neurosci *4*, 151-158.
Yamashita, M. (2008). Synchronous Ca(2+) oscillation emerges from voltage fluctuations of Ca(2+) stores. FEBS J *275*, 4022-4032.

Yanagida, E., Shoji, S., Hirayama, Y., Yoshikawa, F., Otsu, K., Uematsu, H., Hiraoka, M., Furuichi, T., and Kawano, S. (2004). Functional expression of Ca2+ signaling pathways in mouse embryonic stem cells. Cell Calcium *36*, 135-146.

Yi, C.H., and Yuan, J. (2009). The Jekyll and Hyde functions of caspases. Dev Cell *16*, 21-34.

Ying, Q.L., Stavridis, M., Griffiths, D., Li, M., and Smith, A. (2003). Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. Nat Biotechnol *21*, 183-186.

Yuste, R., Nelson, D.A., Rubin, W.W., and Katz, L.C. (1995). Neuronal domains in developing neocortex: mechanisms of coactivation. Neuron *14*, 7-17.

Zitt, C., Halaszovich, C.R., and Luckhoff, A. (2002). The TRP family of cation channels: probing and advancing the concepts on receptor-activated calcium entry. Prog Neurobiol *66*, 243-264.

Zsiros, V., and Maccaferri, G. (2008). Noradrenergic modulation of electrical coupling in GABAergic networks of the hippocampus. J Neurosci *28*, 1804-1815.